Monitoring drug efficacies in equid nematodes

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ABSTRACT

Advances in technology are an important weapon in the fight against anthelmintic resistance. Improved diagnostics are therefore crucial to enable targeted selective treatments and avoid unnecessary anthelmintic use. In common with other grazing animals, equines are at risk of infection with intestinal parasites, and it is crucial that effective control measures are maintained. Evidence suggests the re-emergence of large Strongyles such as *Strongylus vulgaris* given reduced treatment of cyathostomins. Thus, minimising cyathostomin anthelmintic exposure, and controlling the pathogenic large strongyles is a key balancing act.

Faecal egg counts (FECs) are the standard method of diagnosing the level of parasitic infection in horses and other grazing animals. Testing before treatment is an important factor in slowing the appearance of anthelmintic resistance in nematode parasites of horses. The FECPAK^{G2} allows farmers to perform FECs on their own sheep and cattle, without the need for any specialist parasitology knowledge. The current work has optimised the FECPAK^{G2} (G2) method for horses, and validated this against the original FECPAK (G1), using faecal samples from 114 horses in Wales and New Zealand. No significant difference was observed between the FECs obtained using the two methods (rmANOVA: $F_{1,37} = 0.052$, $p = 0.821$, $\eta_p^2 = 0.001$) with no effect noted linked to the country of origin. In addition, the accuracy of the G2 method was not affected by FEC level ($r = -0.251$ (CI: 0.030, -0.472) $p = 0.124$ $n = 39$). Further improvements to the G2 method such as a lower detection limit are possible with planned improvements in the imaging process.

Following validation, the FECPAK^{G2} approach was used to identify anthelmintic resistance to allow the assessment of altering equine nemabiomes pre and post

anthelmintic treatment. DNA from twelve paired faecal samples, pre- and post-treatment with Fenbendazole were sequenced using the MinION (Oxford Nanopore Technologies Ltd.). Six major cyathostomin species were identified across all equine nemabiomes. The most numerous species was *Cyathostomum catinatum* (39% of pre-treatment sequences) with this species displaying consistent resistance to Fenbendazole with 96% still remaining after treatment. It was noted that BZ resistance is potentially linked to predilection site within the GI tract. These findings are consistent with the idea that anthelmintic resistance develops more quickly when sub-lethal doses are administered, and suggests that there is insufficient Fenbendazole remaining in the gut to effectively control helminths towards the end of the gastro-intestinal tract.

FECPAK^{G2} and MinION nemabiome sequencing were also trialled and found to be effective for helminth monitoring in zebra. The work also demonstrated that the cyathostomin nemabiome sequencing method was effective even at low FEC levels. The zebra in this study harboured the same species of cyathostomin as had been found in the equine samples. However, the zebra nemabiomes suggests that the history of repeated BZ administration may have influenced the host nemabiomes towards those species least responsive to BZ treatment.

Given the work conducted the FECPAK^{G2} represents an acceptable method for equine FECs. It is hoped that the user-friendliness of the method will increase the uptake of FECs amongst horse owners, either by direct use of the technology or through their veterinary practice, hence slowing the development of anthelmintic resistance. It was also concluded that MinION sequencing offers a useful method of determining which species of strongyle are present, facilitating a differential diagnosis between more or less pathogenic species and enabling informed treatment

decisions to be made. Interestingly, the entire process of FEC and nemabiome sequencing are portable for use in the field, and thus could be utilised to monitor strongyle infections of exotic equids in wild populations.

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LIST OF ABBREVIATIONS

bp	Base Pairs
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool (nucleotides)
BZ	Benzimidazole
cDNA	Complementary DNA
CTAB	Cetyl Trimethylammonium Bromide
dH_20	De-ionised water
DLP	Dose Limiting parasite
EDTA	Ethylenediaminetetraacetic acid
EL3	Early third larval stage
Epg	Eggs per gram
ERP	Egg reappearance period
FEC	Faecal egg count
FECRT	Faecal egg count reduction test
G1	First Generation FECPAK ($FECPAK^{G1}$)
G2	Second Generation FECPAK ($FECPAK^{G2}$)
GABA	Gamma-Aminobutyric acid
gDNA	Genomic DNA

GI	Gastro-intestinal
HCl	Hydrochloric acid
IVM	Ivermectin
L3	Third larval stage
L4	Fourth larval stage
LL3	Late third larval stage
ML	Macrocyclic Lactone
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
Ng / µl	Nano grams per microlitre
SOP	Standard Operating Procedure
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
P-gp	P-glycoprotein
PYR	Pyrantel
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
SOC	Super Optimal broth with Catabolite repression
THP	Tetrahydropirimidine

TST	Targeted Selective Treatment
WAAVP	World Association for the Advancement of Veterinary Parasitology

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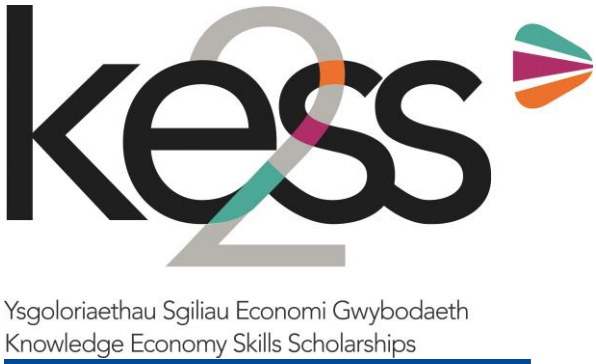

LIST OF PUBLICATIONS

"Worms at the Royal Welsh Show" - Vet Record 181, 376-377.

Tyson, F *et al*. 2020. Novel Equine Faecal Egg Diagnostics: Validation of the FECPAK^{G2}. Animals, 10(8), p.1254.

"Worms in the spotlight" Quarterly column on equine parasite in "Sleipnir" – the magazine of the Icelandic Horse Society of Great Britain.

Promotional literature for Techion Ltd.

One hour lecture for pony club on equine parasites.

One hour lecture for 3rd year Veterinary Biosciences undergraduates on equine parasitology.

Poster presentations at British Society for Parasitology, and European Industrial Doctoral School (first prize poster).

1 INTRODUCTION

1.1 INTRODUCTION

It has long been recognised that horses harbour parasites and since the Roman civilisation this has been identified as a source of disease (Kaplan and Nielsen, 2010). A key group of gastro-intestinal (GI) parasites are the helminths which includes the parasitic nematodes. Therefore, medicinal compounds used to treat them are termed anthelmintics. The first specific anthelmintic compound for horses was Phenothiazine, introduced in the 1940s (Swales, 1942) although reports of resistance to this compound occurred as early as the 1950s (Poynter and Hughes, 1958). The only alternative anthelmintic at the time of resistance emergence, Piperazine, was only 50% effective against species including *Strongylus vulgaris* and was not effective at all against other large Strongyle species (Downing *et al*., 1955). Modern over-the counter anthelmintic compounds appeared on the scene in the 1960s with the introduction of the Benzimidazoles (Kaplan, 2004) with further compound discoveries during the 1970s and 80s. Initially, horse owners were advised to treat all animals routinely every two months with one of these anthelmintics on a schedule designated as interval dosing (Drudge and Lyons, 1966). Where anthelmintics are available the interval dosing approach has been successful in effectively controlling the major large strongyle species *Strongylus vulgaris*, once the most important parasite of managed equines (Kaplan and Nielsen, 2010). However, cyathostomin species, small strongyles, have filled the intestinal niche vacated by the three common *Strongylus* species and are now considered to be the primary nematode parasites of horses (Herd *et al.*, 1981; Lyons *et al.*, 1999). Despite successes, there is recent evidence to suggest a resurgence of the large redworm *S. vulgaris* due to attempts to slow anthelmintic resistance within cyathostomins through reduced application of anthelmintic treatments (Nielsen *et al.*, 2012; Scare *et al.*, 2018b).

Therefore, the equine industry does not currently have the answer to effective and sustainable parasite control.

Parasitic helminth egg shedding is over dispersed in definitive hosts, including horses, with a few individuals shedding the majority of eggs. In common with other pathogens, such as HIV, Malaria and Measles (Galvani and May, 2005), this over dispersion follows the 80/20 rule, where 20% of individuals are responsible for disseminating 80% of the disease (Galvani and May, 2005). The 80/20 rule was effectively illustrated by Laugier *et al*. (2012) who demonstrated that 66.9% of foals infected with *Parascaris equorum* had faecal egg counts (FECs) of 200 epg (eggs per gram) or lower, whereas 14 of the 139 foals had FECs of 1,000 epg or over, up to a maximum of 7,800 epg. Furthermore, in a large U.S. based study, over 70% of horses over 3 years of age were found to be in the low to moderate egg shedding category requiring only two anthelmintic treatments per year (Nielsen *et al.*, 2018b). Despite over dispersion, many owners continue to follow the blanket interval-dosing regimen introduced fifty years previous, treating all animals regularly, likely bimonthly, despite unknown infection levels and worm burdens (Slater, 2017). Such practices are not only wasteful but also hasten the development of anthelmintic resistant parasites as each new generation of parasites is the offspring of those that survived previous anthelmintic treatment (Shalaby, 2013).

Control of parasites, including GI nematodes, is an important part of horse husbandry and is essential to ensure good health and optimum performance. This is especially pertinent given helminth infections have been demonstrated to be the direct cause of 30% of chronic weight loss syndrome in Europe of which 75% were attributed to larval cyathostominosis (Tamzali, 2006). Furthermore, gastrointestinal impaction linked to high Ascarid burdens is a serious health risk for young horses,

with a one year survival rate of typically less than 30% (Cribb *et al.*, 2006). Unfortunately, due to the onset of anthelmintic resistance in *P. equorum*, it can no longer be assumed that such parasites will be controlled with simple routine anthelmintic use (Cribb *et al.*, 2006). Therefore, new sustainable strategies for the control of equine helminths must be developed. However, the presence of cyathostomins was demonstrated to correlate negatively with the risk of colic in an Italian study (Stancampiano *et al.*, 2017) suggesting positives to helminth infections as observed earlier with human infections as part of the hygiene hypothesis (Yazdanbakhsh *et al.*, 2002). Despite the potential benefits, Stancampiano and colleagues suggest that this correlation is likely due to the fact that grazing horses are less likely to suffer colic than stabled horses yet they are also more likely to harbour parasites therefore suggesting correlation but not causation. Alternatively, Stancampiano *et al*. (2017) suggested a possible protective effect of luminal cyathostomins against larval emergence. Either suggestion supports the practice of leaving animals with low helminth infection levels untreated without increasing the risk of disease.

It is frequently advised by the equestrian press (health guidance information) that owners should rotate the classes of anthelmintic within medications in order to delay the appearance of resistance (Elder, 2017). However, such suggestions are despite research evidence that such an approach is not effective (Uhlinger and Kristula, 1992). Moreover, this problematic advice further leads to complacency, with many owners following programmes of anthelmintic rotation and routine treatment in the absence of diagnostic testing that delineates if these treatments are either necessary or successful (Relf *et al.*, 2011). Furthermore, slow rotation (alternating classes of anthelmintic on an annual basis) also remains a recommendation to support the delay

in development of resistance (Kaplan, 2002). In contrast, computer modelling of helminth control strategies suggests that treating simultaneously with two different anthelmintic classes could be the most effective method (Kaplan, 2002). However, a case study testing the proposed Kaplan hypothesis observed that after the initial increased efficacy, likely due to elimination of single-anthelmintic resistant parasites, the efficacy of a combined treatment with oxibendazole and pyrantel pamoate decreased with subsequent doses (Scare *et al.*, 2018a). Furthermore, Scare *et al*. concluded that due to the lack of effective anthelmintic classes available for use in equids the combination approach is likely to be less useful in horses than in ruminant species.

Anthelmintic control of helminths in horses is likely to be enhanced through effective pasture management including removing faeces from grazing areas, which results in a lower infection rate (Tzelos *et al*., 2017). A combined strategy of correct targeted anthelmintic administration and pasture cleanliness offers the most sustainable prospect for continued helminth control in the future (Coles, 2002).

1.2 ANTHELMINTICS AND ANTHELMINTIC RESISTANCE

Anthelmintic resistance in nematode parasites is proposed to develop in a similar manner to antibiotic resistance in bacteria with frequent dosing and sub-lethal doses applying a selection pressure that increases anthelmintic resistant alleles (World_Health_Organisation, 2019). Thus, a chemotherapeutic that is not 100% effective will kill most individual pathogens but leaves those resistant to its activity to reproduce and produce the next generation of individuals. An important method to mitigate this is to leave a population of nematode parasites untreated, or "in *refugia*". The *refugia* population can comprise nematode parasites in animals harbouring only

low levels of infection, nematode parasite life-stages that are unaffected by the anthelmintic utilised and nematode parasites located outside the treated animal, such as eggs or free-living stages on the pasture (Nielsen *et al.*, 2007) (Figure 1-1).

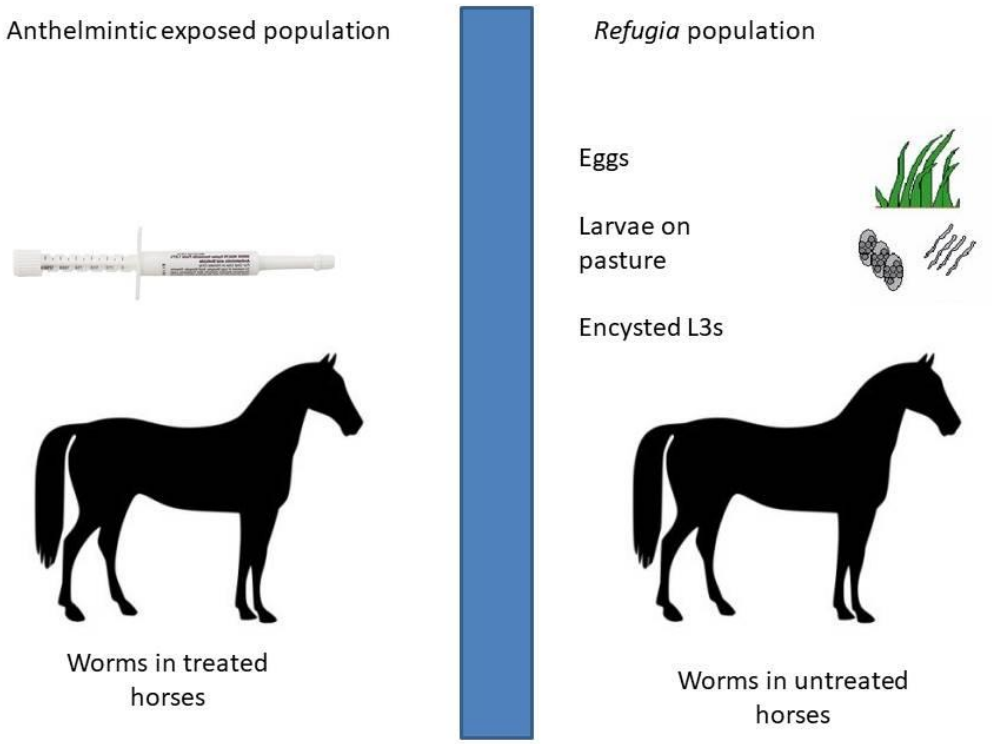

Figure 1-1 Anthelmintic exposed nematode parasites vs Refugia population of parasites (NB encysted L3s are included in anthelmintic exposed population in the case of Moxidectin treatment).

Importantly, when using an anthelmintic such as Ivermectin or Moxidectin, which have a high level of effectiveness in cyathostomins, treating only horses with an infection level over 200 epg can reduce total egg shedding in the herd by 96%, with 98% of these eggs being shed by nematodes in *refugia* within untreated horses, substantially reducing the selection pressure for resistance (Figure 1-2). Thus, maintenance of a *refugia* population helps to preserve the susceptible alleles within the overall parasite gene pool.

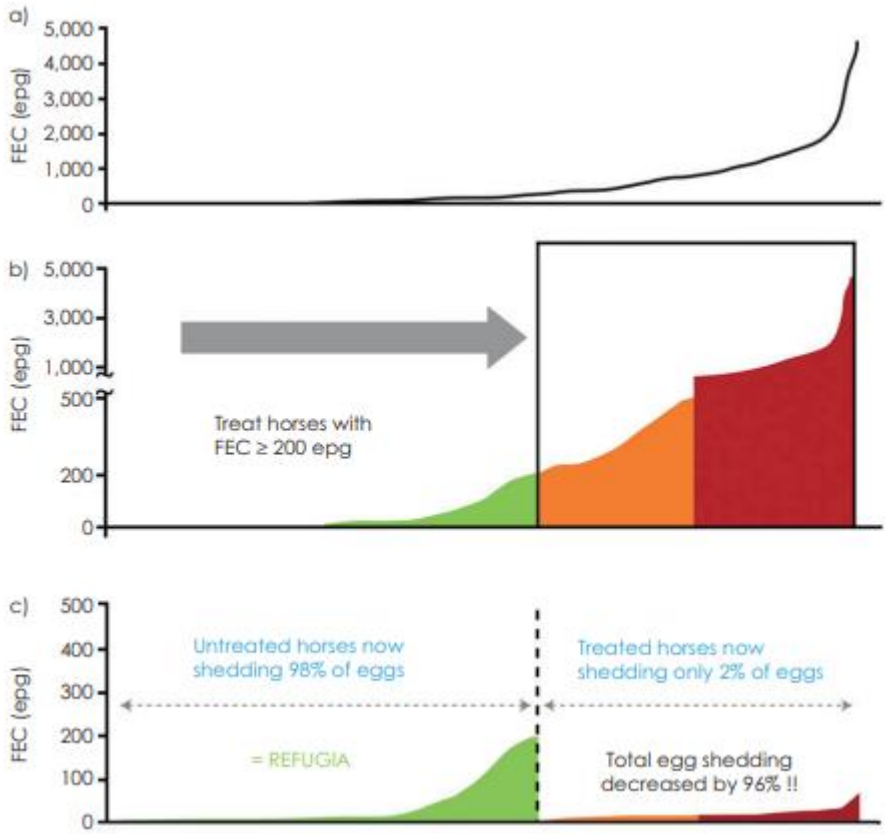

Figure 1-2 The effect of selective treatment on the *refugia* population of nematode parasites in horses adapted from Kaplan and Nielsen (2010). a) FECs from horses on 12 farms (n = 261) plotted in FEC magnitude order from left to right, with FEC on the y axis and individual horses on the x axis. It demonstrates the over-dispersion of egg shedding, with most horses having low FECs and only a few having very high FECs. b) Replication of the FEC data in a) but with the y axis broken for better visualisation of the data. Horses are divided into low egg shedders (green: under 200 epg), moderate egg shedders (orange: 200 – 500 epg) and high egg shedders (red: over 500 epg). c) The expected FEC distribution after treatment with an anthelmintic with high efficacy (99.9% e.g. Ivermectin or Moxidectin). Total egg shedding is decreased by 96% despite only half the horses being treated and 98% of eggs are now being shed by nematodes in *refugia* in untreated horses.

There are currently three major classes of anthelmintic used to control nematode parasites in horses: benzimidazoles (BZ; example active compounds Fenbendazole and Oxibendazole), Tetrahydropirimidines (THPs; active as Pyrantel salts), and macrocyclic lactones (ML; example active compounds Ivermectin and Moxidectin). In addition to nematode control, the cestocide Praziquantel is used for the control of tapeworm only, and although not licensed for use in horses, the flukicide

Triclabendazole, given the increase in fluke infections (Quigley *et al.*, 2017), can be used off-label where necessary to control liver fluke in horses. These anthelmintics are however outside the scope of this review.

The mode of action of the Benzimidazole anthelmintics is quite well understood. Micro-tubules which form the cytoskeleton of all eukaryotes are formed of dimers of α- and β- tubulins, and BZ anthelmintics work by disrupting isotype 1 of the β-tubulin gene (Gilleard, 2006) which inhibit polymerisation of the tubulins and hence the formation of the microtubules, damaging the cell structure and killing the parasite (Lacey, 1990). THPs like Pyrantel work by disrupting nematode neuro-transmitters. They bind to receptors for acetyl-choline on the body wall muscles, which causes spastic paralysis in the nematodes, resulting in them being expelled from the host (Abongwa and Martin, 2017). Macrocyclic lactones also act on nematode neuro-transmitters, in this case glutamate-gated chloride channels, causing paralysis and blocking pharyngeal pumping, with Ivermectin also being an antagonist for 4-aminobutyric acid (GABA) and nicotinic receptors which are expressed on the somatic muscle cells of parasitic nematodes (Abongwa and Martin, 2017).

It is often quoted in the scientific literature that parasite populations which become anthelmintic resistant are unlikely to revert to susceptibility (Jackson *et al*., 1998; Sangster, 1999). However, a summary of *P. equorum* anthelmintic resistance to the three major classes of anthelmintics documented in the literature demonstrate that, along with an increase in resistance to Ivermectin, there seems anecdotally to be a return to efficacy of benzimidazoles, particularly Oxibendazole (Table 1-1). It should be noted that the two studies noting resistance to Pyrantel (Lyons *et al.*, 2008; Lyons *et al.*, 2011) were both accounts from the United States, where use of PYR tartrate

daily de-wormer is common practice. Such an apparent return to efficacy of the BZ anthelmintics is statistically demonstrated only (i.e. it is not specifically the same population of parasites in all studies). Nevertheless, it is reassuring that there is at least one class of anthelmintic that remains reasonably effective against *P. equorum* (90% or over at the time of writing), albeit that evidence for this is anecdotal.

It is thought that reversion to susceptibility is not long-lived, as nematodes with alleles for resistance quickly multiply upon reintroduction of the anthelmintic (Leathwick, *et al.* 2001). However, a recent study in sheep, using combinations of different classes of anthelmintics in conjunction with methods to maximise *refugia* populations of parasites demonstrated an unexpected return to efficacy of anthelmintics that had previously been ineffective due to resistance (Leathwick *et al.*, 2015). Therefore, as recommendations for the anthelmintic treatment of horses aim to maximise *refugia* populations (Nielsen *et al.*, 2007) this offers hope that careful management strategies could extend anthelmintic efficacy even after the development of resistance.

Table 1-1 Summary of anthelmintic efficacies against *P. equorum* noted in the literature, demonstrating a development of resistance to Ivermectin (IVM) and simultaneous reversion to susceptibility to Benzimidazoles (BZ).Pyrantel (PYR) data included for completeness.

Paper	Year	IVM efficacy	PYR efficacy	BZ efficacy
Vandermyde *et al.*	1987			As 5 day course only
DiPietro *et al.*	1987	100%		
French *et al*.	1988	100%		
Austin *et al*.	1991	98.2%	74.2%	44.5%
Gawor	1996			50%
Boersema *et al*.	2002	No	Yes	
Hearn and Peregrine	2003	No		
Lyons *et al.*	2006	Low		High
Lyons *et al.*	2007 Published 2008	Zero	Very little	94%
Slocombe *et al*.	2007	33.5%	97.6%	97.6%
Molento *et al.*	2008	95%	94%	100%
Veronesi *et al.*	2009	63% (2 farms)	100%	
Lind and Christensson	2009	Very low	Over 90%	Over 90%
Veronesi *et al.*	2010	ineffective	effective	
Näreaho *et al*.	2011	52%		
Lyons *et al*.	2011		2%	80% & 97%
Bishop *et al.*	2013 Published 2014	0% - 69%		
Beasley *et al*.	2015	65%		

It is noticeable that different nematode species are developing resistance to different classes of anthelmintic suggesting that in order to treat both ascarids and cyathostomins successfully in young horses it is necessary to use more than one broad spectrum compound. In support, during a study in Kentucky in 2007 BZ wormers were effective against ascarids but not against cyathostomins, whereas the reverse was true for IVM (Lyons *et al.*, 2008). Furthermore, PYR was not found to be effective against either species (Lyons *et al.*, 2008). Thus, using any single class

of anthelmintic exclusively would potentially fail to control both species of nematode and risk failing to control either species.

In addition, in Brazil only Fenbendazole was fully effective against *P. equorum* and none of the MLs gave adequate control to cyathostomins. Furthermore, none of the three classes of anthelmintic were effective at 28 days post-treatment (Molento *et al.*, 2008). Thus, multi-drug resistance is a serious potential problem and must be guarded against by strategic application of anthelmintic compounds.

As most anthelmintic compounds are broad spectrum, the dose needed to control different species of nematode may not be the same. The nematode species which requires the highest dose to be effective is generally known as the Dose Limiting Parasite (DLP). The ascarid species *P. equorum* is often the DLP in horses for most anthelmintic compounds (Reinemeyer, 2009). Therefore, the ascarid parasites present are more likely to receive a dose only just effective in concentration, whereas other species are receiving a dose which is more than sufficient. This will likely have the effect of hastening the development of resistance in the DLP compared to other species (Reinemeyer, 2009). Fortunately, *P. equorum* is rare amongst equid parasites in that most host animals develop absolute acquired immunity to infection over time, meaning that infection is a problem only in young animals (Clayton and Duncan, 1979; Reinemeyer, 2009). Nevertheless, as ascarid infection can be fatal in foals and weanlings (Laugier *et al.*, 2012) it is important to control these parasites. Therefore it is particularly imperative to ensure that the correct dosages of anthelmintics are administered to populations of foals and young horses accounting for the bodyweight of the animals. This will likely ensure that under-dosing is avoided and delay the development of anthelmintic resistance (Matthee, 2003).

Moreover, exploiting anthelmintics which are less effective (for example, efficacies of 80% rather than 98%) may surprisingly delay the development of resistance, while retaining a measure of parasite control (Coles, 2002). Conversely, anthelmintics such as Moxidectin, that target all developmental stages of a parasite rather than leaving encysted larvae as a *refugia* population, will likely cause resistance to develop more rapidly (Coles, 2002) as there will be no new generation of untreated worms to appear when the larvae excyst. Such effects are somewhat mitigated by the fact that the efficacy of Moxidectin against encysted larval stages of cyathostomins is only 63.6% (EL3) and 85.2% (LL3/L4) respectively (Reinemeyer *et al.*, 2015) meaning that there is the potential for a substantial number of treatment survivors to emerge and form a new parasite population.

The previously recommended practice of treating animals and then moving them to clean pasture also hastens the development of resistance as all larvae deposited on the pasture will have been exposed to anthelmintic treatment, so it is recommended that this be discontinued (Coles, 2002). However in a regime of targeted, selective treatment (TST) where only high egg shedding animals are treated with anthelmintics, the movement of horses to clean pasture after treatment is less problematic as most nematode eggs deposited on the pasture will come from untreated animals (Kaplan and Nielsen, 2010).

Frequency of treatment with anthelmintic compounds increases the speed with which resistance develops. PYR resistance in cyathostomins is more common in farms in the U.S.A. where a small daily dose of Pyrantel tartrate is fed (Kaplan, 2002). Paradoxically, this daily treatment leads owners to believe that their horses are fully protected against nematode parasites, hence regular sufficient doses of anthelmintics

are rarely given. Thus, those horses in greatest need of parasite control are actually receiving the poorest treatment (Kaplan, 2002).

In order to prolong the efficacy of anthelmintic compounds, it has been recommended to test for resistance against all anthelmintics used, only treating animals with a FEC of 200 epg or greater (see Table 1-2) and testing horses introduced into a yard for IVM resistance before allowing them to graze with others (Coles, 2009).

Table 1-2 Equine FEC levels and action required (Westgate Laboratories, 2020)

	FEC level	**Action**
Low	<200epg	No action required
Medium	200 – 1199 epg	Treat with anthelmintic
High	<1200 epg	Treat with anthelmintic and review worming / pasture control programme.

In addition, it is recommended to collect manure from the pasture, weekly in winter and twice weekly in summer (Coles, 2009), to cross graze with sheep where possible and not to graze foals on pasture used by foals the previous year (Coles, 2009). It has also been suggested that treating only those animals with physiological signs of infection, in addition to maximising *refugia* populations and delaying the development of resistance, could also select for those endoparasites that minimise their pathological effect on the host (Beech *et al.*, 2011). Development of anthelmintic resistance is significantly reduced in premises where targeted treatment

plans based on FECs are implemented (Sallé *et al.*, 2017) underlining the importance of FEC testing as an aid to parasite control.

1.3 LARGE STRONGYLES

When modern anthelmintics first became available during the 1960s, the major nematode parasites of clinical importance in horses worldwide were the large Strongyles, primarily *Strongylus vulgaris*, with prevalence reported worldwide of between 80 and 100% (Nielsen *et al.*, 2012). After having been ingested by the horse, the larvae of large strongyles penetrate the gut wall, and migrate through the blood vessels to the heart – causing oedema of the gut mucosa, and dilation of the small arteries, veins and capillaries (McCraw and Slocombe, 1976). Thrombosis can occur in the ilieo-caeco-colic and cranial mesenteric arteries due to migrating fourth stage larvae with infiltration of neutrophils (Duncan and Pirie, 1972), and thickening of the cranial mesenteric artery and its branches due to fibrin tracks which become overgrown with endothelium (Duncan and Pirie, 1972). With the introduction of the first anthelmintics, and the subsequent wholesale adoption of the interval-dosing regime (Drudge and Lyons, 1966), *S. vulgaris* has become rare in managed horse populations (Herd *et al.*, 1981). As a result, *S. vulgaris* has now been superseded as the principle nematode parasites of importance in infections of horses, by the small Strongyles, also known as the cyathostomins (Herd *et al.*, 1981; Lyons *et al.*, 1999).

1.4 CYATHOSTOMINS (SMALL STRONGYLES)

Cyathostomins are considered less pathogenic than large strongyles as the former do not migrate parenterally but only through the mucosa and occasionally the submucosa (Lyons *et al.*, 1999). However, they are unique among parasites of horses in that they can enter a state of arrested development at the third larval (L3) stage

and encyst in the intestinal mucosa (Eysker *et al.*, 1984). The cyathostomin lifecycle has alternate development options for infective L3 larvae ingested from the pasture, which may develop into adults in the lumen of the intestine, or enter a period of arrested development as encysted larvae in the gut wall (Figure 1-3).

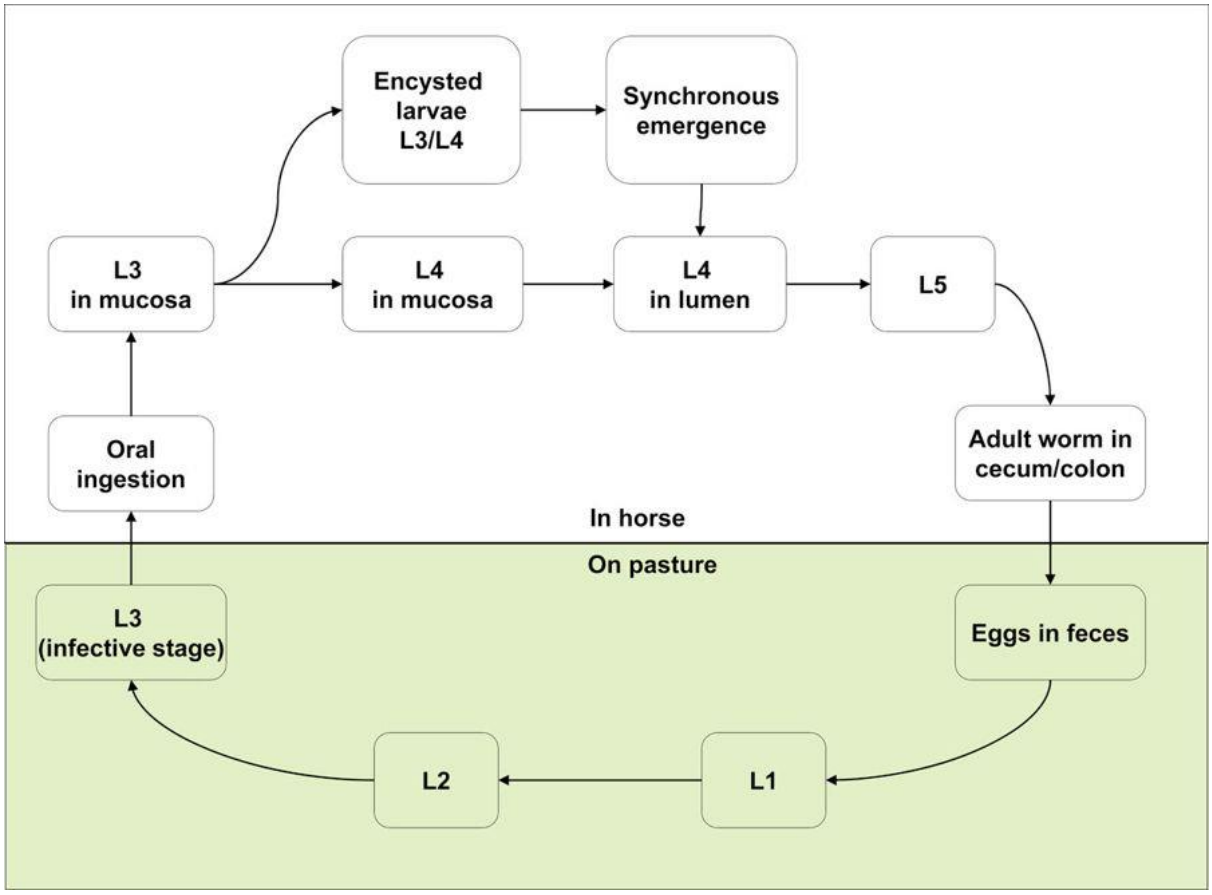

Figure 1-3 Lifecycle of cyathostomins. Alternate options for ingested infective L3 larvae are available including the development of L3 larvae directly into L4 larvae or L3 larvae encystment in the gut wall (Corning, 2009).

Cyathostomins are pathogenic at all life stages. In addition to the nutritional demands of large infections of adult worms, the fact that the early L3 stages invade the gut wall and encyst can cause serious damage to the gut mucosa, compromising the horse's ability to absorb nutrients from its food (Collobert-Laugier *et al.*, 2002). After entering the gut wall, the early L3 mature into late L3 and then L4 larvae which then emerge *en masse* back into the lumen of the gut. Emergence of large numbers of encysted cyathostomin larvae from the gut wall can cause larval cyathostominosis (Love *et al.*, 1999) resulting in severe diarrhoea and weight loss in addition to colic, which can be fatal in as many as 50% of cases (Stratford *et al.*,

2011). As such, cyathostomins became recognised as important equid parasites (Love *et al.*, 1999).

A heavy infestation with adult and encysted L4 cyathostomins can cause neutrophilia, hypoalbuminaemia, hyperglobulinaemia, in particular beta-globulin, consistent with a protein-losing enteropathy (Corning, 2009). On a cellular level, histopathology shows an inflammatory response to cyathostomin larvae involving mononuclear cells, eosinophils and epithelial cells (Abbott, 1998).

1.4.1 Cyathostomin anthelmintic resistance

There is widespread resistance to BZs amongst cyathostomins recognised across the globe (Peregrine *et al.*, 2014) such that these anthelmintics are rarely used as a routine control measure (Slater, 2015). Furthermore, although to a lesser extent, widespread resistance of cyathostomins to PYR exits globally, along with evidence of developing resistance to MLs (Peregrine *et al.*, 2014). Worryingly, resistance to all three classes of anthelmintic has been discovered within the UK (Traversa *et al.*, 2009).

Currently the most common anthelmintics administered by horse owners are the MLs: Ivermectin and Moxidectin (Slater, 2015). However, following the later introduction of Moxidectin there are fears that its widespread use is likely to hasten the development of resistance to Ivermectin in cyathostomins. Such suggestions are based upon the action of Moxidectin on the encysted larval stages which represent a *refugia* population when treated with Ivermectin (Sangster, 1999). A reduced egg reappearance period (ERP) is considered to be a precursor to the development of anthelmintic resistance (Shea Porr *et al.*, 2017). Recent work has observed a reduced ERP for Moxidectin of four to five weeks as opposed to the 16 – 22 weeks

historically reported when the anthelmintic was first introduced (Bellaw *et al.*, 2018). Both ML anthelmintics are known to target a group of glutamate-gated Cl^- channels within parasitic nematodes and therefore, given the similarity in their mode of action, resistance to one compound may also confer resistance to the other (Sangster, 1999).

A recent computer modelling study predicted that reducing anthelmintic treatments by selectively treating horses would reduce the development of anthelmintic resistant nematode populations, and that this effect was greater in temperate climates like the UK (Nielsen *et al.*, 2019). This hypothesis was borne out by a Danish study, which observed Ivermectin to be 100% effective against cyathostomins, likely a direct consequence of the country's policy of restricting anthelmintic use to those horses identified by a veterinary surgeon as requiring treatment (Larsen *et al.*, 2011). This illustrates the potential success of a policy of targeted treatment only as a means of preserving anthelmintic efficacy.

Owing to the potentially serious nature of larval cyathostominosis, owners are recommended to administer an anthelmintic effective against encysted larvae, either a five day course of BZ or a single dose of Moxidectin, in the autumn or early winter (Westgate Laboratories, 2018). Despite such advice for a BZ or Moxidectin based approach, recent studies have reported resistance to the five day BZ regime (Bellaw *et al.*, 2018; Steuer *et al.*, 2018) leaving only Moxidectin as an effective treatment against encysted cyathostomin larvae. However, the comparative efficacy of a five day course of BZ against early L3s at two and five weeks post treatment was found to be 50.4% and 51.3% respectively with Moxidectin only showing 73.8% and 71.8% efficacy (Bellaw *et al.*, 2018) suggesting that neither treatment regime is particularly effective. At the time of writing a diagnostic blood test to identify an

encysted cyathostomin burden in horses has just been introduced (BVA, 2019) , and work is underway to develop a saliva based test ((Mitchell *et al*., 2016)). Better diagnostics will inform treatment decisions against encysted cyathostomin larvae, avoiding un-necessary anthelmintic treatments for these parasite life stages thus helping to delay the development of anthelmintic resistance.

1.5 PARASCARIS EQUORUM

A nematode from the family *Ascaridae*, *Parascaris equorum*, is an important parasite of foals (Clayton and Duncan, 1979). *P. equorum* follow the recognised hepatic-tracheal route of migration within the definitive host (Figure 1-4).

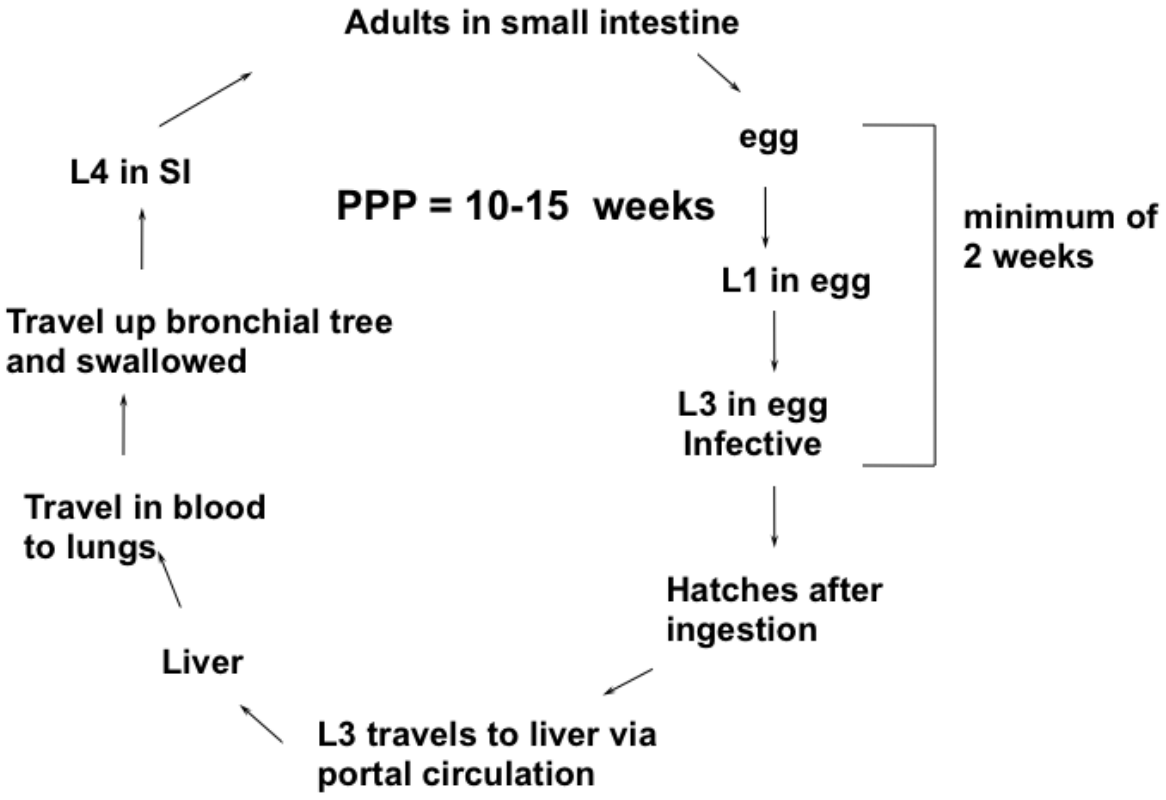

Figure 1-4 Lifecycle of *P. equorum* (www.studyblue.com), showing hepatic tracheal migration of larvae. PPP = Prepatent Period, SI = small intestine

Adult *P. equorum* lay eggs in the small intestine of infected horses and these are passed out with the faeces. The larvae develop within the eggs and undergo two moults become infective, larvated (L3), after approximately ten days and can remain in this state on the pasture for five to ten years (Lindgren *et al*., 2008). Foals and young horses ingest these infective eggs whilst grazing, and the eggs then hatch and migrate through the liver and the lungs for approximately one month. From the lungs, *P. equorum* larvae are coughed up and swallowed to reach their predilection site in the small intestine and mature into adults reaching patency at 75-80 days after infection (Clayton, 1986).

Equids usually build up immunity to *P. equorum* from 5-6 months of age so infection is rare in adult horses (Laugier *et al.*, 2012). Infected foals can suffer from respiratory symptoms, stunted growth, ill-thrift, rough coat, diarrhoea and colic (Clayton and Duncan, 1978). A heavy *P. equorum* infection can cause intestinal blockage or rupture from large numbers of adult worms. In addition, as a consequence of larval migration, lesions in the liver, fibrosis and infiltration by oeosinophils and lymphocytes can occur (Brown and Clayton, 1979). As infection in older horses is rare, foals become infected through ingesting eggs from the pasture, deposited by previous generations of foals (Schougaard and Nielsen, 2007). For this reason, foals at stud farms are at particular risk, as out of necessity they are grazing land that foals have grazed previously.

1.5.1 *Parascaris equorum* anthelmintic resistance

In a critical study in 1991, it was observed that Ivermectin had 98.2% effectiveness against *P. equorum*, PYR pamoate 74.2% and Oxibendazole only 44.5% effectiveness (Austin *et al.*, 1991). However, Table 1-1 demonstrates that these figures are not consistent in different regions and have not remained constant over

time. *P. equorum* are highly fecund and produce a large quantities of eggs (Clayton, 1986), which are extremely robust and can survive on pasture for many years. As these eggs represent a substantial *refugia* population and ML anthelmintics, such as Ivermectin, are highly effective against the luminal stages of the parasite, it was once thought that resistance was unlikely to develop (Boersema *et al.*, 2002). It has therefore become common practice for stud farms to dose all foals routinely with Ivermectin as the sole method of control of these parasites. Unfortunately, this has not proven to be the case, with reports of resistance appearing very shortly afterwards (Boersema *et al.*, 2002).

The first report of possible *P. equorum* resistance to MLs occurred in the Netherlands (Boersema *et al.*, 2002). Foals on this farm were routinely dosed with Ivermectin at 10 days, three weeks and six weeks and then bimonthly thereafter. The authors suggested that such frequency of treatment had contributed to the resistance observed, despite the previous belief that *P. equorum* were unlikely to develop resistance (Boersema *et al.*, 2002). Resistance to Ivermectin was then reported in Canada in 2002 and 2003 (Hearn and Peregrine, 2003; Slocombe *et al.*, 2007) and in Kentucky, Germany and Denmark in 2004 (Lyons *et al.*, 2006; Schougaard and Nielsen, 2007; von Samson-Himmelstjerna e*t al.*, 2007b) and Sweden in 2006 (Lindgren *et al.*, 2008). A later Danish study found that Ivermectin had 96.9% efficacy against *P. equorum.* However, the mean age of horses in this study was 2.1 years, meaning that there was a possible confounding effect of acquired immunity (Larsen *et al.*, 2011).

The first report of a UK infection with Ivermectin resistant *P. equorum* came in 2006 with the death of a foal from severe *P. equorum* infection despite anthelmintic treatment every four weeks with Ivermectin (Stoneham and Coles, 2006). Additional

foals from this farm were subsequently successfully treated with a five day course of Fenbendazole. In 2008 *P. equorum* resistant to Moxidectin, yet susceptible to PYR, were discovered by a UK veterinary surgeon in a horse imported from the Netherlands (Peaty, 2008) and, in a letter published in answer to this report, a representative of Fort Dodge Animal Health (manufacturers of the Moxidectin based anthelmintic "Equest") stated that MLs were not the anthelmintic of choice for use against *P. equorum* (Traill, 2008). Despite this advice, the data sheet for "Equest" stated that it is effective against *P. equorum* (Zoetis Ltd, 2013) although this has now been amended to refer to Moxidectin sensitive strains (Zoetis Ltd, 2019).

Ivermectin resistance in *P. equorum* was later observed in other European countries (Veronesi *et al.*, 2010; Veronesi *et al.*, 2009); (Lind and Christensson, 2009); (Laugier *et al.*, 2012; Näreaho *et al.*, 2011) and in New Zealand and Australia (Beasley *et al.*, 2015; Bishop *et al.*, 2014). Furthermore, *P. equorum* populations resistant to PYR were found in Kentucky (Lyons *et al.*, 2011). A 2014 study of foals on Australian stud farms detected resistance to all three classes of anthelmintic in *P. equorum*, with some farms having multi-drug resistance populations. Fortunately, none of the farms tested showed resistance to all three classes simultaneously (Armstrong *et al.*, 2014). These studies illustrate that monitoring of anthelmintic efficacy against *P. equorum* is essential to ensure effective treatment of these nematodes.

1.6 NEMATODES AND ANTHELMINTIC RESISTANCE IN SPECIFIC EQUID POPULATIONS

1.6.1 Donkeys

Donkeys can be asymptomatic vectors of the nematode lungworm *Dictyocaulus arnfeldi* which has been known to infect horses (Soulsby *et al.*, 2004). In horse infections, *D. arnfeldi* cause severe coughing and breathing difficulties often accompanied by chronic pneumonia and secondary bacterial infections. Patent infections in adult horses do not occur, so only those horses co-grazed with donkeys are at risk (Soulsby *et al.*, 2004). In addition, in common with horses, donkeys can suffer from large Strongyles, Cyathostomins and *P. equorum* (Soulsby *et al.*, 2004).

Resistance to all three classes of anthelmintic compounds, including triple-drug resistance, has been found in cyathostomins infecting donkeys (Lawson *et al.*, 2015). Targeted treatment plans can be hampered where large numbers of donkeys are kept together, such as the Donkey Sanctuary (Sidmouth, U.K.) which has over 3,000 resident donkeys (Soulsby *et al.*, 2004), making it impractical to perform FECs on each individual animal. In contrast, anthelmintic resistance in the parasites of donkeys is rarer in the developing world, as the use of anthelmintics is decreased owing to lack of availability and refractive costs (Soulsby *et al.*, 2004). Cyathostomin ERP was found to be shorter in donkeys (42-55 days) than in horses following Moxidectin treatment (Matthee *et al.*, 2002), suggesting that greater care needs to be taken with donkey herds to prevent the development of full-scale anthelmintic resistant parasites.

1.6.2 Mules

Relatively few studies have looked at the mule population. However, research in Mexico on working equids demonstrated that mules were more likely to be infected with nematode parasites than horses or donkeys (Valdéz-Cruz *et al*., 2006). Mules also displayed higher levels of infection than either horses or donkeys when assessed via FEC epg (Valdéz-Cruz *et al.*, 2006). Of the few studies that have investigated parasite levels in mules, the results are not always reported separately from results obtained from horses (Foster, 1937; Pereira and Vianna, 2006) making it difficult to draw comparisons for this group of equids.

1.6.3 Zebras

Free ranging wild zebra have typically higher worm burdens than do managed animals and in the absence of anthelmintic treatment are still commonly infected with large strongyles, which are now rare in domestic horse populations (Wambwa *et al.*, 2004). A nematode commonly observed in both ranched and free ranging zebra is *Crossocephalus viviparous* from the family *Atractidae* which does not appear to infect horses even when sharing the same habitat (Krecek *et al.*, 1995) and is often found in high levels (Wambwa *et al.*, 2004).

The majority of cyathostomin species infect all equids. However, there are some species particular to one host such as *Cylicocyclus triramosus* which is known to infect only zebra (Kharchenko *et al.*, 1997). Confirmation using scanning electron microscopy has revealed that the ascarid species infecting both wild and captive zebra is the same one that infects the domesticated horse, namely *P. equorum* (Ansel *et al.*, 1974).

1.7 METHODS OF NEMATODE DETECTION

1.7.1 Faecal egg counts

A number of methods of performing faecal egg counts (FECs) exist, which are useful in determining the presence and level of nematode egg shedding and hence which horses should be treated. In all methods, samples of fresh faeces are diluted in a flotation solution and examined microscopically to count the number of eggs per gram (epg) present. Historically, the most commonly used FEC method was the McMaster technique (MAFF, 1986). The McMaster method has been adapted to include counting the entire McMaster slide chamber in an attempt to increase sensitivity (Torgerson *et al.*, 2012). However, due to egg aggregation at the centre, of the slide it is actually more accurate to count only the grids as intended by the designers (Godber *et al.*, 2015). Although many flotation solutions can be used, a saturated saline solution (specific gravity 1.20) should be sufficient to float all three commonly found species of helminth eggs in equine samples – strongyles (specific gravity 1.05), the tapeworm *Anoplocephela perfoliata* (specific gravity 1.06) and *Parascaris equorum* (specific gravity 1.09) (Norris *et al.*, 2018).

One recommendation is to use the FECPAK version one system (FECPAK^{G1}), as it is more sensitive (25 epg) than the McMaster method (50 or 100 epg), which was originally developed for sheep but adapted for equids (Coles, 2009). The FECPAK^{G1} system utilises a microscope slide in a similar manner to that of the McMaster test but allows for a larger volume of faeces to be examined, thus improving sensitivity and rendering the test able to estimate mean egg density more accurately over a wide range of infection levels (Presland *et al.*, 2005). Additional FEC methods that are more sensitive than the McMaster such as the Mini-FLOTAC (10 epg) or FLOTAC

(1 epg) are also preferable, particularly where egg counts are low (Levecke *et al.*, 2012b; Rinaldi *et al.*, 2014).

1.7.2 Use of technology in FECs

In November 2017 an alternative automated FEC system, the Poop2Proof system (later renamed Parasight), launched onto the market, initially for Veterinary surgeons only (MEP_Equine_Solutions, 2019). This system analyses faecal samples using fluorescent staining of nematode eggs followed by imaging and analysis via computer aided programmes. However, the performance of the system is questionable, having an overall accuracy of only 32.5% when tested on spiked samples (Scare *et al.*, 2017). Of particular concern is the fact that the system only detected 25% of the eggs in samples spiked at 500 epg and 29% of the eggs in samples spiked at 1000 epg. In reality, this suggests that a treatable infection (>200 epg) would likely not have been diagnosed until the actual infection level was 1000 epg (Scare *et al.*, 2017).

1.7.2.1 FECPAK^{G2} method

A system that is currently in development for equines, the FECPAK^{G2}, aims to produce automated FEC images without the need for a separate microscope. This system will have a similar sensitivity (26 epg) to its predecessor the FECPAK^{G1} method, and will produce images that can be stored or transmitted via the internet (Unpublished data, 2016). The FECPAK^{G2} system is currently in use for sheep and cattle (Techion Ltd, Pers. comm), it has been tested in alpacas (Rashid *et al.*, 2018) and is also being optimised for use in humans (Ayana *et al.*, 2018). Although in its infancy for equine faecal samples, the FECPAK^{G2} system was successfully used in a small study at the Royal Welsh agricultural show which found that horses whose

owners did not include FECs in their parasite treatment regime had FECs over four times higher than horses who received FECs (Tyson *et al.*, 2017).

Nevertheless, the use of technology in developing new FEC methodologies, particularly those that require little in the way of specialist knowledge, should encourage the use of FECs as part of a parasite management programme in domestic horses and other species. Such engagement across the equid sector is necessary as recent surveys demonstrate that FECs are still not widely used in the equine industry, with a recent study reporting only 22% of establishments using FECs at all and only 10% using them on a regular basis (Nielsen *et al.*, 2018a).

1.7.3 Faecal egg count reduction tests

The most accurate method of studying levels of parasitic helminth infection in livestock and hence anthelmintic resistance is termed 'dose and slaughter' (Schougaard and Nielsen, 2007). However, as horses are not regularly slaughtered for consumption in the UK, critical studies in which the subjects are necropsied to determine the level of parasitic infection are uncommon. Therefore, the faecal egg count reduction test (FECRT) is considered to be the most effective technique for measuring anthelmintic resistance in equids (Kaplan, 2002). In brief, one FEC test is performed on the day of treatment and a further FEC performed fourteen days after treatment with the reduction in eggs recorded signifying the efficacy of the anthelmintic delivered. A reasonable and often applied definition of anthelmintic resistance would be: i) percentage reduction in egg count less than 95% and ii) lower limit of 95% confidence level less than 90% (Coles *et al.*, 1992). These criteria were proposed by the World Association for the Advancement of Veterinary Parasitology

(WAAVP) in order to define anthelmintic resistance in 1992. More recently, it has been suggested that different resistance alert levels should be designated depending on the anthelmintic actually utilised (Kaplan, 2002). To this end, the American Association of Equine Practitioners (AAEP) parasite control guidelines advise that BZ resistance should be assumed with FECRT of <90%, THP resistance with <85% and ML resistance with <95% (Nielsen *et al.*, 2013). However, research comparing different methods of assessing FECRTs suggests that current WAAVP guidelines for assessing anthelmintic efficacy, as described in Coles *et al*. (1992), remain the most appropriate (Levecke *et al.*, 2018).

Researchers are currently aware that significant caution must be taken in interpreting the results of a FECRT since an apparent reduced efficacy of the anthelmintics administered can simply be due to the sensitivity of the FEC method used, as well as a potentially incorrect or ineffective dosing (El-Abdellati *et al.*, 2010). Further confounding factors for FECRT interpretation can be the relative level of aggregation of infection within the host population and often the alarming absence of a direct relationship between the level of nematode infection and the level of egg excretion (Levecke *et al.*, 2012a). However, this said, the FECRT remains the most utilised and effective tool for determining anthelmintic resistance levels in equine nematode parasites.

Thus, in order to test accurately for decreased anthelmintic efficacy, it is desirable to exploit the most sensitive test available, e.g. the FLOTAC technique which is sensitive to 1 epg (Coles, 2009). This is clearly important where infection levels can imply a relatively low burden (Levecke *et al.*, 2011). However, this method is time-consuming and technically demanding, requiring 11 separate steps including two centrifugations (Levecke, 2016 personal conversation). In field situations, where

resistance is present, as defined by the WAAVP, all tests trialled, including a modified McMaster with a sensitivity of 10 epg, have been found to be effective at detecting anthelmintic resistance (Levecke *et al.*, 2012b). Furthermore, where either egg counts are high or an approximate count is needed to determine whether or not treatment is required, then any of the FEC tests would be appropriate (Bosco *et al.*, 2014). Thus whilst highly sensitive FEC methods may be required for research purposes, routine diagnostics can be accomplished using tests with lower diagnostic sensitivity.

An alternative method, rather than highlighting the mean FEC reduction from a number of animals, is to treat the egg as the experimental unit, rather than the horse (Dobson *et al.*, 2012). Performing repeat counts on high shedders until, for example, 200 eggs have been counted (actual eggs seen rather than computed epg figures) can also be used instead of a single count from each animal available (Levecke, 2016, personal conversation). Dobson *et al.* (2012) also recommended the use of Jeffreys confidence intervals to estimate the precision of the FECRT in cases where anthelmintic efficacy is estimated to be 100%, as other statistical methods cannot generate confidence intervals under such circumstances (Dobson *et al.*, 2012). Jeffreys confidence intervals should only be used when efficacy is 100% however, not under any other circumstances, where they may distort results (Levecke, 2016, personal conversation).

In sheep, it has been demonstrated that using pooled samples from several animals is as effective as using individual FECs for the estimation of mean level of infection and for testing anthelmintic efficacy (Rinaldi *et al.*, 2014). However, due to the level of over dispersion of helminth parasite burdens in horses (Laugier *et al.*, 2012), it

may be a less useful method in this species. Nevertheless, pooled sample testing has been found to be beneficial for pre-screening in horses (Eysker *et al.*, 2008).

1.8 MOLECULAR GENETICS

1.8.1 Diagnostics and species identification

Molecular tests are now available to support comprehensive diagnostics by both quantifying and speciating the parasitic nematode populations infecting a host animal, termed the 'nemabiome'. This was initially achieved in cattle, although also applicable to horses, by culturing nematode larvae from the eggs in the host faeces and undertaking Next Generation Sequencing (Avramenko *et al.*, 2015). Further research in equines has realised the possibility of performing such tests directly on faecal samples, thus saving the time consuming larval culture step (Mitchell *et al*., 2019) Furthermore, molecular genetic techniques have been employed for determining the change in the nemabiome following anthelmintic treatment in cattle (Avramenko *et al.*, 2017).

1.8.2 Detection of anthelmintic resistant nematodes

As long ago as 2004 it was stated that there was an urgent need to develop and validate molecular assays for the detection of anthelmintic resistant nematodes in the field in order to replace treatment failure (phenotypic detection) as the sole option (Kaplan, 2004). One route towards a molecular assay is to explore the genetic basis of anthelmintic resistance by embracing the candidate gene approach. Thus, based on our understanding the mode of action for each anthelmintic, we can identify the genes involved and potentially identify mutations in these genes that occur in resistant nematodes. This approach has been successful in identifying the isotype-1 β-tubulin locus as the most important indicator of BZ resistance in a number of

parasitic nematode species (Gilleard, 2006) across ruminants and equids. It is not thought that the isotype-2 β-tubulin locus is implicated in BZ resistance in cyathostomins, as no polymorphisms have been consistently observed in the analysis of resistant and susceptible populations (Hodgkinson *et al.*, 2008). The role of the β-tubulin 200 codon mutation is not as strongly linked with the resistance phenotype in cyathostomins as in other helminths such as the trichostrongylids (Coles *et al.*, 2006; Hodgkinson *et al.*, 2008). However, a non-synonymous A/T polymorphism causing and amino acid shift at residue 167 is likely to be implicated in BZ resistance in Cyathostomins (Drogemuller *et al.*, 2004; Pape *et al.*, 2003; von Samson-Himmelstjerna *et al.*, 2007a). In a 2008 study, homozygous mutations causing amino acid substitutions at either residue 200 or residue 167 conferred BZ resistance yet, in 158 parasites genotyped, none had homozygous mutations at both loci underlying these key residues. This suggests that a double mutation may be lethal (Hodgkinson *et al.*, 2008). Recent work in Brazil has also identified a potential SNP in the codon encoding for residue 172 which results in a serine to threonine substitution that may also be related to BZ resistance (Ishii *et al.*, 2017).

The candidate gene approach is far more complex in the case of Ivermectin resistance, as a number of different gene mutations have been associated with the resistance phenotype (Gilleard, 2006). Under these circumstances a genome-wide approach would be more successful once whole genome data are available for equine nematodes (currently genomes for the cyathostomin *Cylicostephanus goldi* and the large strongyle *Strongylus vulgaris* are available on Wormbase Parasite). Nevertheless, a number of genes have been identified that are associated with resistance to different classes of anthelmintic (Table 1-3).

Table 1-3 Genes associated with nematode resistance to four different classes of anthelmintic. (Beech *et al.*, 2011).

For each gene, the molecular change associated with the appearance of anthelmintic resistance is indicated, along with the type of diagnostic test typically used to identify mutations associated with resistance: SNP-PCR, PCR based test that identifies specific single nucleotide polymorphism as indicated in the text; QT-PCR, quantitative PCR that can estimate the relative abundance of different RNA transcripts, PCR, indicates a test where the specific size of a PCR product, or the presence or absence of a specific product forms the basis of the test. AchR = Acetylcholine Receptor, Glu/GABA channel = Glutamate or GABA gated channel

Anthelmintic	Target	Resistance gene	Molecular change	Molecular test
BZ	beta-tubulin	β-tubulin	F200Y	SNP-PCR
			E198A	SNP-PCR
			F167Y	SNP-PCR
LEV	AchR	unc-38	Decreased expression	QT-PCR
		unc-63	Altered transcript	PCR
		acr-8	Altered transcript	PCR
MPTL	AchR	mptl-1	Altered transcript/decreased expression	PCR/QT-PCR
		des-2	Altered transcript/decreased expression	PCR/QT-PCR
		deg-3	Altered transcript/decreased expression	PCR/QT-PCR
IVM/MOX	Glu/GABA channel	avr-14	L256F	SNP-PCR
		lgc-37	K169R	SNP-PCR
		glc-5	A169 V	SNP-PCR
		ggr-3	Decreased expression	QT-PCR
		pgpA	Increased expression	QT-PCR

Thus far, although polymorphisms in ML anthelmintic-uptake receptors have been identified in several studies, no mutations have yet been identified which explain the

resistance found phenotypically in parasites in the field (Kotze *et al.*, 2014). It is therefore likely that the mechanism of resistance in the field is multi-genic (Kotze *et al.*, 2014). Recent work has strongly implicated P-glycoproteins (P-gps), components of Phase III detoxification, as being at least partly responsible for reduced sensitivity to Ivermectin (Peachey *et al.*, 2017) raising the possibility of the use of P-gp inhibitors to enhance the efficacy of the anthelmintic and reverse resistance. Furthermore, although causality has not been established, a link has been observed between survival of ML treatment and BZ resistance suggesting that treatment with MLs could also select for BZ resistant nematodes (Blackhall *et al.*, 2008; de Lourdes Mottier and Prichard, 2008).

Genes for anthelmintic resistance may occur in one of four ways. Mutations conferring resistance may be ancient, pre-existing the start of anthelmintic use; new mutations could arise immediately before, or during, the period of anthelmintic use; mutations may occur recurrently; or they may be introduced as a result of a new animal being brought to the premises (Gilleard and Beech, 2007). Genetic diversity in parasitic nematodes is high, due to their large population size and high mutation rate (Nadler, 1987). It has been demonstrated that this diversity includes alleles for resistance even before the first exposure to anthelmintics (Coles *et al.*, 2005). Applying a high selection pressure has been demonstrated to produce anthelmintic resistant *Haemonchus contortus*, an important parasitic nematode of sheep, in only three generations (Coles *et al.*, 2005).

In order to obtain anthelmintic resistant parasites for genetic analysis, one of two methods are required to be employed. Firstly, it is necessary either to produce resistant strains experimentally in the laboratory, or to harvest naturally resistant parasites on farms where resistance has previously been confirmed. Laboratory

selection typically incorporates lower doses of anthelmintics than would be administered in the field as sufficient parasites are required to remain alive in order to produce successive generations. Therefore, resistance alleles produced under laboratory conditions may not have a major effect or, more importantly, may not reflect the infield situation (Gilleard and Beech, 2007). However, resistant parasites obtained from the field are harder to compare with non-resistant strains as there may be substantial underlying genetic variability not connected to anthelmintic resistance (Gilleard and Beech, 2007). An added layer of complexity is due to the diversity of cyathostomin species that infect horses, which number more than 50 with a typical infection consisting of more than five different species (von Samson-Himmelstjerna, 2012), sometimes many more (Mitchell *et al*., 2019).

As noted previously, molecular genetic techniques have demonstrated that there is a correlation between ML exposure and an increase in the codons implicating resistance to benzimidazoles (de Lourdes Mottier and Prichard, 2008) suggesting that use of MLs could predispose parasites to BZ resistance. Such a predisposition has serious implications for anthelmintic rotation regimes, as anthelmintic resistance alleles could be selected for even in the absence of drug exposure. In addition, if anthelmintic resistance occurs due to the evolution of efflux mechanisms in parasitic nematodes, multi-drug resistance could easily occur (James and Davey, 2009).

In the sheep sector, DNA based testing is able to identify benzimidazole resistant strains of *H. contortus*, allowing alternative anthelmintics to be used instead (Kotze *et al.*, 2014). However, it is questionable whether such tests would be useful in horses as widespread knowledge of resistance to BZ anthelmintics in cyathostomins means that they are seldom used routinely (Slater, 2015).

A recent study using reverse line blot observed that this technique was a useful semi-quantitative method for detecting different cyathostomin species in pooled samples - a substantial reduction in work from individual testing. Reverse line blot thus represents a potentially suitable method for differentiating the species present in groups of horses, although less accurate for larval culture of a single horse (Kooyman *et al.*, 2016). Next generation PCR techniques could also be used for such assays, and an investigation into the equine nemabiome, and how this changes before and after anthelmintic treatment, could yield useful information about the exact species of cyathostomin most resistant to treatment. This technique has already been successfully deployed in cattle (Avramenko *et al.*, 2017). It has been observed that fewer than ten horses need to be sampled in order to determine the species composition of cyathostomins at a single premises (Sallé *et al.*, 2018). There is some evidence that the developmental stage of strongyle eggs may affect the quantity of DNA present and hence the efficacy of PCR assays (Andersen *et al.*, 2013). Therefore, it is important to determine which tests will be performed on a faecal sample, in order to determine the optimal storage conditions.

It has been suggested that, rather than using molecular techniques to identify resistant strains of parasite, detecting those that are susceptible to anthelmintic treatment would instead be a useful practice (Beech *et al.*, 2011). Assays to predict anthelmintic susceptibility would inform treatment decisions and indicate the most useful anthelmintic treatment to apply, rather than guesswork followed by testing for resistance.

1.8.3 Reappearance of *S. vulgaris*

A side effect of targeted anthelmintic treatment in the equid sector is the reappearance of *S. vulgaris*, once considered to be virtually eliminated in managed

horses (Nielsen *et al.*, 2012). In a Danish study, it was noted that 2% of colic cases admitted to a veterinary hospital were due to non-strangulating infarctions caused by *S. vulgaris* (Pihl *et al.*, 2017). Over ninety percent of these cases were fatal, including 100% of cases that weren't treated surgically (Pihl *et al.*, 2017). It is impossible to differentiate large Strongyle species, such as *S. vulgaris*, from less pathogenic cyathostomin species using egg morphology (Lichtenfels *et al.*, 2008b). Previously, larval hatch assays were required to enable species diagnosis from faecal samples, yet as genetic sequencing becomes more available, the potential for species identification from eggs becomes possible. Using molecular techniques as a diagnostic aid, targeted anthelmintic treatments could potentially include differential diagnosis between *S. vulgaris* and cyathostomin infections, thus helping prevent serious disease.

1.9 CONCLUSION

It is clearly of the utmost importance that each premises monitors the susceptibility of its helminth parasite populations to different classes of anthelmintics. At present, such an aim in nematode parasites is best achieved by FECRTs, although molecular genetics may yield more efficient methods in the near future. New methods of detecting anthelmintic resistance in *P. equorum* are particularly necessary as the FECRT method has not been validated in this species, neither has a correlation between high FECs and a high worm burden been demonstrated (Reinemeyer, 2009).

In addition to ensuring that effective chemicals are used, dosing frequency should be reduced as far as possible. Thus, targeted selective treatment plans based on dosing only those horses with the highest parasite burdens will help balance the need to avoid parasitic disease and pasture contamination, with the absolute essential of

reducing the development of anthelmintic resistance. Historically, a figure of 200 epg has been used as the level at which treatment should be given, but it has been suggested that raising this to 500 epg would help delay the development of resistant worms (Molento *et al.*, 2008; Veronesi *et al.*, 2009). As the relationship between parasite burden and pathology is not precisely known, treating only those horses shedding high levels of eggs or those showing signs of parasitic disease is recommended (Coles, 2002). Finally, non-chemical approaches to avoiding parasite infection, such as removal of dung regularly from pasture, must be regarded as essential for 21st Century equine management, as such measures are correlated with reduced parasitic infection (Tzelos *et al*., 2017).

1.10 AIMS

The aims of this PhD are to support the development of new technologies in the monitoring of infection level and anthelmintic resistance in nematode parasites of horses. Firstly, given that the FECPAK^{G2} faecal egg counting platform has not previously been evaluated for the equid sector, the FECPAK^{G2} will be assessed and optimised for use in horses. Furthermore, the FECPAK^{G2} will be validated against currently recognised and accepted FEC methods. It is envisaged that by demonstrating that the diagnosis of nematode parasite infection is simpler and easier through the application of technology, owners will be encouraged to monitor infection level and treatment efficacy. Importantly, moving away from a blanket interval dosing treatment regimen will help delay the development of anthelmintic resistance and moreover, diagnostics are essential in a move toward targeted selective treatment (TST).

Anthelmintic resistance in a number of horse establishments will be monitored by performing FECs both before and after treatment. During this phase, samples will be retained and DNA extracted. Next Generation DNA sequencing will be carried out to investigate the equine nemabiome and how this changes in the face of treatment with anthelmintics. It is hoped that this relatively simple, non-invasive method of discovering which species are present and which are more resistant to anthelmintic treatment, will increase knowledge of the equine nemabiome, and provide a "proof of concept" that will inspire further studies of how this changes in various circumstances.

Aims:

- Assess and optimise FECPAK^{G2} for equine samples (Chapter 2).
- Validate optimised FECPAK^{G2} protocol for equine samples (Chapter 3).
- Assess level of anthelmintic resistance in areas of the UK and monitor nemabiome shift in the face of anthelmintic treatment (Chapter 4).
- Investigate application to exotic equids (Chapter 5).

2 DEVELOPMENT OF THE FECPAK^{G2} FOR EQUINE USE

2.1 INTRODUCTION

The fundamental component for successful Targeted Selective Treatment (TST) of nematode parasites in horses is a diagnostic test to identify which animals are the high egg shedders that should be selected for anthelmintic treatment. Faecal egg counting or FEC remains the mainstay of diagnostic testing for nematode parasites. Importantly, studies show that the use of FECs as a management tool can lead to lower egg counts (Tyson *et al.*, 2017) and thus less need for anthelmintic treatment, yet other studies demonstrate that the majority of horse owners do not utilise FECs at all, or do not perform them with the recommended frequency (Slater, 2017). It is hoped that by improving the ease with which FECs can be performed, horse owners will be encouraged to use them more regularly.

2.1.1 The FECPAK^{G2} system

The FECPAK^{G2} system (Techion Ltd) is currently commercially available for counting gastro-intestinal nematode eggs in sheep faeces. This system involves the owner performing simple preparation steps on a sample of faeces, then loading it into an imaging device which captures an image and uploads it via the internet where it can be evaluated by trained technicians. The egg count is then electronically returned to the owner, together with support on deciding whether or not to treat the animal with anthelmintics. The problem to overcome in developing the FECPAK^{G2} system for equine use is that the volume and consistency of equine faeces differs significantly from that of sheep faeces (Fritz *et al.*, 2009), which, coupled with the potentially different level of nematode egg shedding in horses, requires each stage of the FECPAK^{G2} preparation of the faecal samples to be optimised to ensure that the FECPAK^{G2} test will perform acceptably for the new host species. Therefore, it was

hoped to devise a standard operating procedure (SOP) for equine faecal sample preparation so that the FECPAK^{G2} could successfully be used to perform egg counts on nematodes in horse faeces. This approach would have the dual benefit of providing a novel method of faecal egg counting for the equine sector, and additionally opening up a new market for the Techion FECPAK^{G2} system.

The original FECPAKG2 protocol for sheep samples is as follows:

- Mix faeces with water in the ratio 1:3 and mix to form a uniform slurry
- Spoon 12 ml slurry into a sedimentor and fill with water, invert three times to mix
- Allow to stand for 30 minutes, then discard the supernatant
- Add 80 ml saturated NaCl solution to the sediment and pour into a FECPAK^{G2} cylinder fitted with 600 µm and 425 µm (sliver) filters, invert three times to mix
- Fill both wells of the FECPAK^{G2} cassette from the cylinder, mixing between each aliquot
- Allow the cassette to stand for six minutes for the eggs to accumulate, then image using the Micro-I

Initial work using equine samples in the Techion Ltd laboratory had determined that the initial dilution of the sample needed to be 1:4 to produce a faecal slurry of the required consistency. In addition the liquid in the sedimentor needed to be filtered through a 1mm mesh prior to the sedimentation to remove large debris. These two steps were added to the sheep protocol when working with equine samples.

2.2 CHAPTER AIMS

- Devise a SOP for the FECPAK^{G2} for use with equine samples

2.3 OPTIMISATION OF THE FECPAK^{G2} FOR EQUINE USE

The optimisation steps which were followed are outlined in Figure 2-1. These steps were included in order to determine if equine faeces would behave in a different way to sheep faeces in the different stages of the FECPAK^{G2} preparation and to ensure that any differences in the faecal consistency and the concentration of nematode eggs therein did not introduce errors into the test.

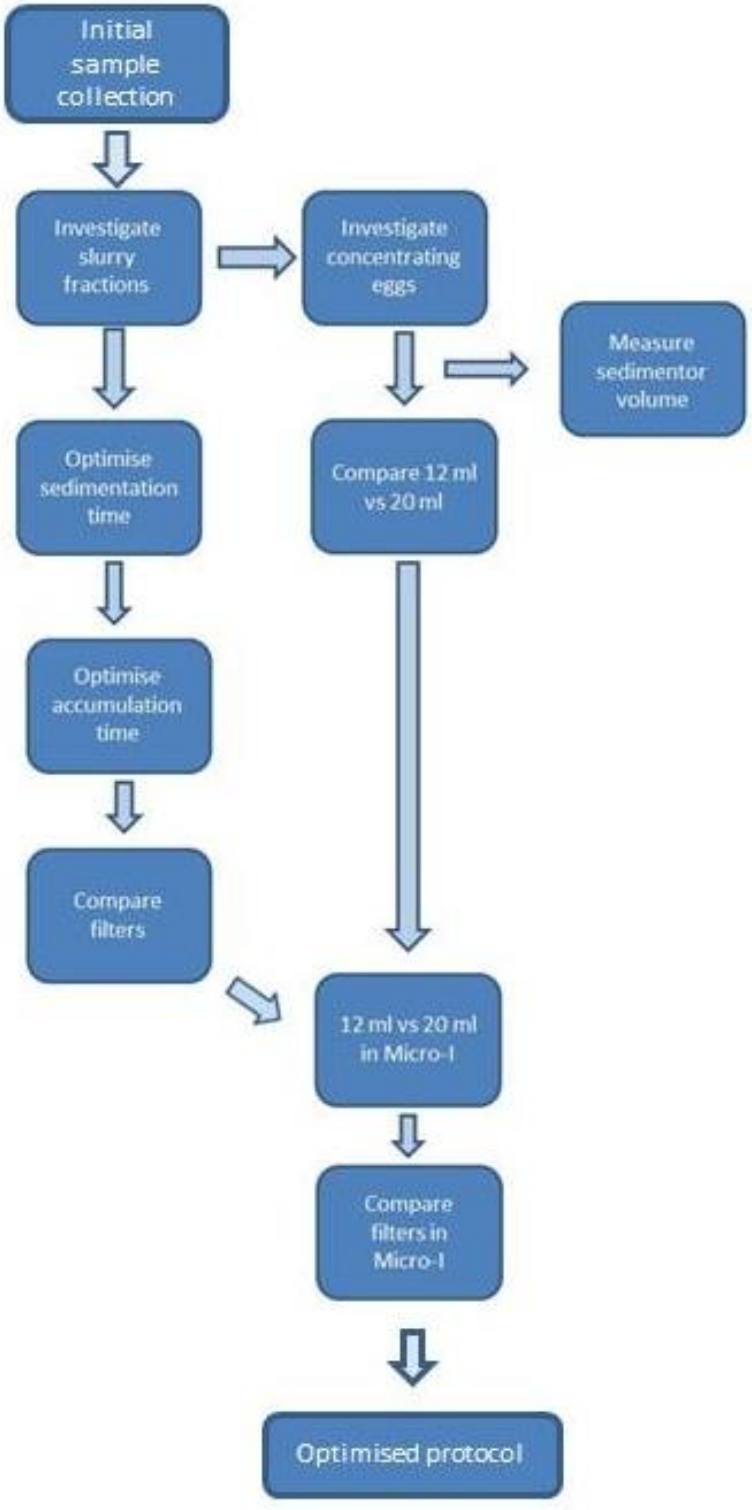

Figure 2-1 Optimisation steps undertaken in order to determine the best protocol for processing equine samples for the FECPAK^{G2} Sedimentation refers to the process whereby the faeces are diluted with water and left to stand in a sedimentation device, until the nematode eggs and heavier debris have settled to the bottom, and any lipids or light debris have floated to the top where they can be poured off and discarded. Accumulation refers to the process of allowing the nematode eggs to float to the surface of the flotation solution, prior to being imaged by the imaging equipment – the FECPAK^{G2} Micro-I.

2.4 INVESTIGATING SLURRY FRACTIONS

2.4.1 Slurry fraction investigation - introduction

In order to optimise the FECPAK^{G2} for equine use, the initial optimisation was focused on investigating the initial sampling of the faecal slurry so as not to introduce error into the FEC process through the dilution of the faecal sample. Following dilution of the faeces with water, a faecal slurry is produced, which by its nature is a mixture of small particles suspended in liquid, and thus may provide varying FECs dependent on the sampling method chosen.

Prior to the development of the FECPAK^{G2} (FECPAK second generation, or G2), Techion Ltd. marketed FECPAK (protocol in Appendix 7.1), a faecal egg counting methodology, and subsequently referred to as the FECPAK^{G1} (G1) to distinguish it from the FECPAK^{G2}. The initial step of both the G1 and the G2 Techion Ltd. methods for equine samples involved diluting the faecal sample in a 1:4 ratio with water in a Ziploc bag (Figure 2-2). The diluted sample was recommended to be poured from the Ziploc bag into the receptacle for the next stage of the sample preparation protocol (45 ml slurry in the case of the G1 method and a reduced volume of 12 ml slurry in the case of the G2 method).

Figure 2-2 Ziploc bag used for initial sample dilution showing closure mechanism which was trapping the solid matter from the faeces. (www.togopackaging.com)

It was noted that when dispensing a large volume of slurry for the G1 method, the initial watery fraction was followed by more solid matter. However, in the case of the G2 sample, requiring a reduced volume of slurry, the zip of the Ziploc bag trapped any solid matter and, coupled with the small volume dispensed this meant that the initial watery sample was not immediately followed by solid material. Thus this process for the G2 gave cause for concern that the resulting aliquot of faecal slurry would not be representative of the sample as a whole, both the liquid and the solid fractions.

In sheep, for which both the FECPAK methods were originally developed, the faeces produced a uniform faecal slurry as ruminant digestion breaks the food matter down into very small particles (Fritz *et al.*, 2009). However, as hindgut fermenters, horse

faeces generally contain much larger particles of solid matter (Fritz *et al.*, 2009). Consequently, in the initial step of both the G1 and G2 preparation, it proved impossible to produce a faecal slurry which did not separate into watery and more solid matter, despite how well mixed the faeces and water had been. Given the challenges with non-uniform faecal slurry it is likely that nematode eggs maybe released into the watery fraction, with the risk of over-stating the egg count, or retained in the solids potentially under-stating.

2.4.2 Slurry fraction investigation - materials and methods

Faecal slurry preparation was replicated on three different biological samples, once on a sample with a high FEC and twice on more average ones. For each faecal slurry, 20 g of faeces from a horse with a natural strongyle infection were mixed with 80 ml water in a Ziploc bag. The resulting slurry was mixed until it was of uniform consistency. Twenty grams of faeces and 80 ml of water gave a workable quantity of slurry whilst ensuring that a larger sub-sample of faeces were used compared to the standard McMaster method (MAFF, 1986). The use of a larger sub-sample is an important improvement, as it helps to mitigate the effects of nematode egg aggregation within the faecal pile (Denwood *et al.*, 2012).

Following faecal slurry generation, slurry was poured from the Ziploc bag into the Techion Ltd. sedimentor (see Figure 2-3) up to the slurry line (12 ml), followed by 80 ml of saturated NaCl solution. In this range finder experiment, the samples were not sedimented, as the goal was to determine the distribution of eggs in the slurry.

Figure 2-3 Techion Ltd. FECPAK^{G2} sedimentor, highlighting 12 ml slurry line.

The sedimentor lid was fitted and the sedimentor inverted three times to mix the slurry and NaCl solution. The slurry and NaCl solution was then pre-filtered using a 1,000 µm filter then poured into a FECPAK^{G2} filter cylinder fitted with a 600 µm and a 425 µm filter, (Figure 2-4) the two standard filter sizes used for the FECPAK^{G2} sheep protocol. The cylinder was inverted three times to mix the solution well, and an aliquot used to fill a FECPAK^{G1} slide, which was counted using light microscopy at 40 × magnification.

Figure 2-4 FECPAK^{G2} cylinder and filter system. a) shows the two filters and the cylinder separately, b) shows the filters nested inside each other and fitted within the cylinder.

The remaining faecal slurry was remixed in the Ziploc bag, and treated as before until all slurry was used. The consistency of slurry used for each preparation was noted.

In addition, further technical replicates were performed as above except that faecal slurry was spooned into the Techion Ltd. sedimentor up to the slurry line. As above, faecal slurry post preparation was imaged under light microscopy at 40 × magnification this time counting two slides from each preparation. A control value for each spooned sample was also obtained by making a standard G1 preparation and counting it four times to provide a mean value.

2.4.3 Slurry fraction investigation - results

The initial step of the FECPAK methodology involves diluting the faecal sample with water to produce a slurry. In equine samples, this slurry is not uniform, due to the fibrous nature of horse faeces. Instead, the slurry separates into a watery fraction

and a more solid fraction, regardless of how well mixed it is. In order to ensure that the initial slurry step did not introduce error into the sample preparation, five replicates were undertaken. The first three replicates were prepared by pouring the slurry into the sedimentor, which resulted in the first sedimentors prepared using the watery fraction of the slurry, and the later ones using the more solid fraction.

Replicate one (Figure 2-5a) used a sample from a highly infected horse (3550 epg). The first five sub-samples were watery in consistency, and sub-samples six to eight were more solid. The egg counts clearly demonstrate that for this sample the watery fraction of the slurry (Figure 2-5a blue data points) had fewer eggs in it than the solid fraction (Figure 2-5a red data points).

Replicate two (Figure 2-5b) used a sample from a horse with a more average infection level (228 epg). This animal was chosen in case the results from the first replicate were due to the very high infection level. This sample yielded seven sub-samples, with the first four being watery and the next three being more solid. The experiment was inconclusive, due to the third sub-sample returning an unusually high result. It was possible that this was just due to an outlier, so a third replicate was made using a sample from another horse with an average infection level (266 epg).

Replicate three (Figure 2-5c) again produced eight sub-samples, with the first four being watery, the fifth being of intermediate consistency, and sub-samples six to eight being more solid. This sample did not produce any outliers, and generated comparable results to replicate one in that there were more eggs contained in the solid part of the slurry than in the watery part.

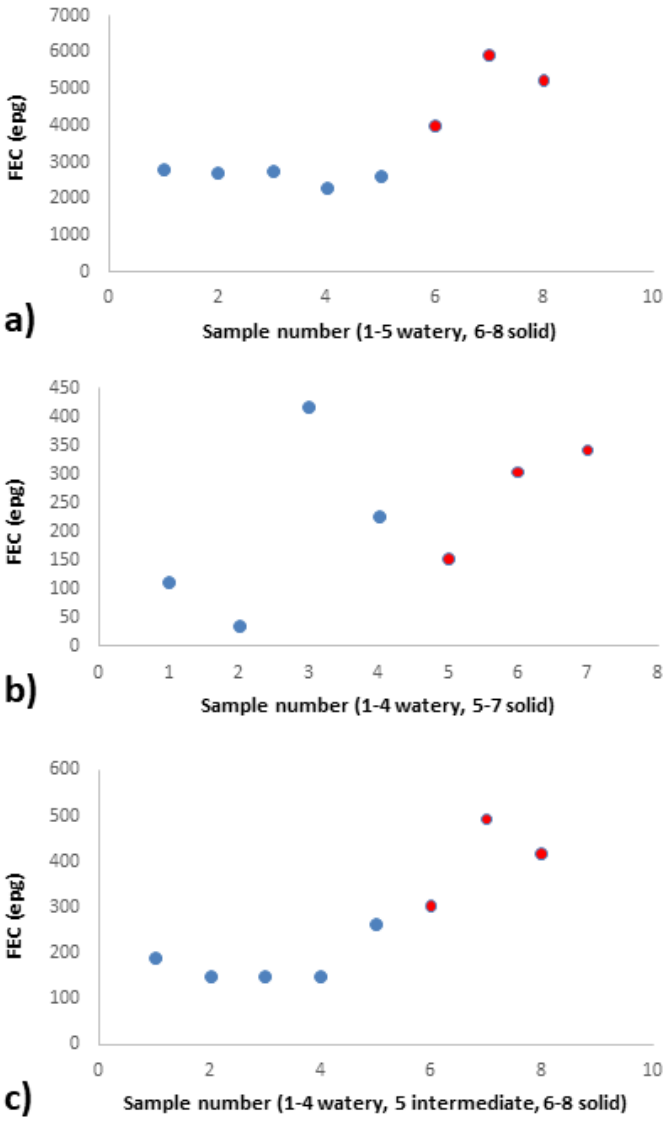

Figure 2-5 Comparing different slurry fractions. a) using high epg faecal sample, b) and c) using average epg faecal samples. Watery sub-samples shown as blue data points, solid sub-samples shown as red data points. Egg recovery in solid fractions up to double that of watery fractions (Sub-sample 5 in c) was intermediate consistency between watery and solid)..

Two more replicates were prepared by spooning the well-mixed slurry into the sedimentors, ensuring as representative a sub-sample as possible.

Replicates four (Figure 2-6a) and five (Figure 2-6b) demonstrated that when the slurry was stirred and spooned into the sedimentor ensuring that the slurry was of an

even consistency across all the subsamples, the repeat counts were more consistent with each other rather than rising with each subsequent aliquot used.

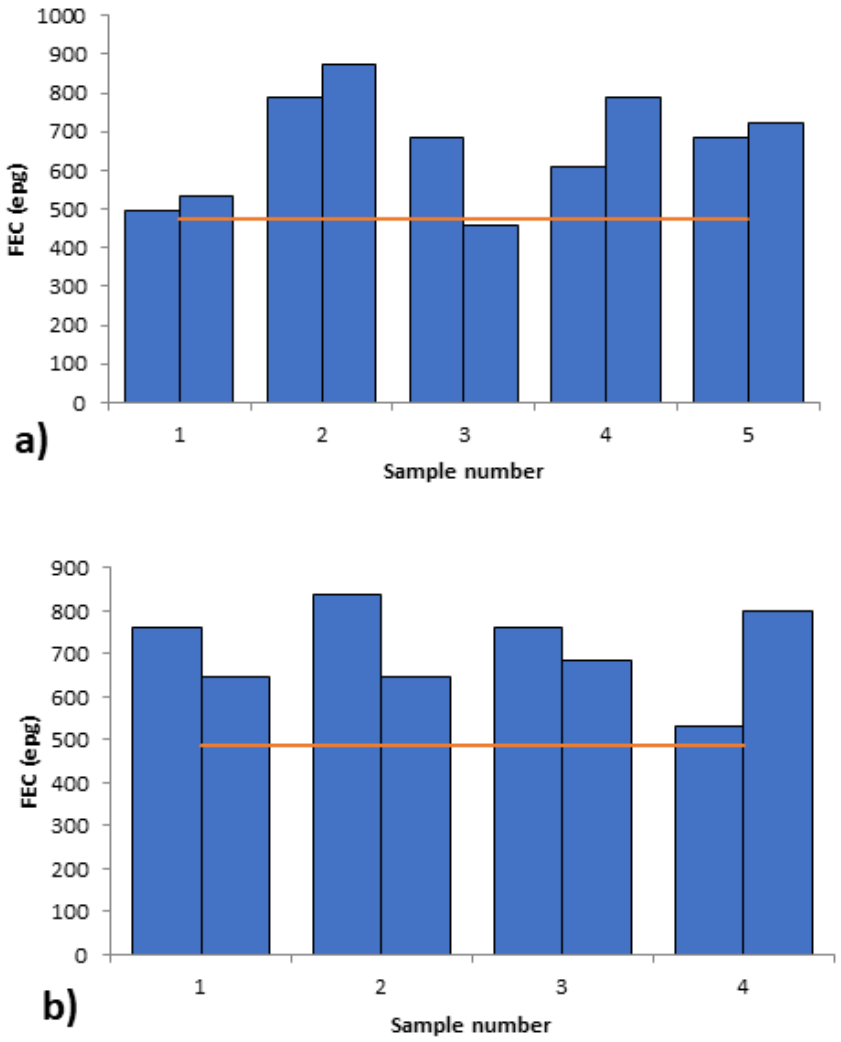

Figure 2-6 Repeated counts from spooned sample a) replicate 4 and b) replicate 5, both using uniform sub-samples of the faecal slurries. Control value is the mean of four G1 counts, shown by yellow line

.

The data from replicates four and five were re-presented to demonstrate the separate G1 counts rather than the mean of the four counts (Figure 2-7a and Figure 2-7c), and only the first count from each spooned sub-sample (Figure 2-7b and Figure 2-7d).

The repeated spooned sub-samples, which contained both watery and solid matter, gave repeatability similar to that of the G1 counts.

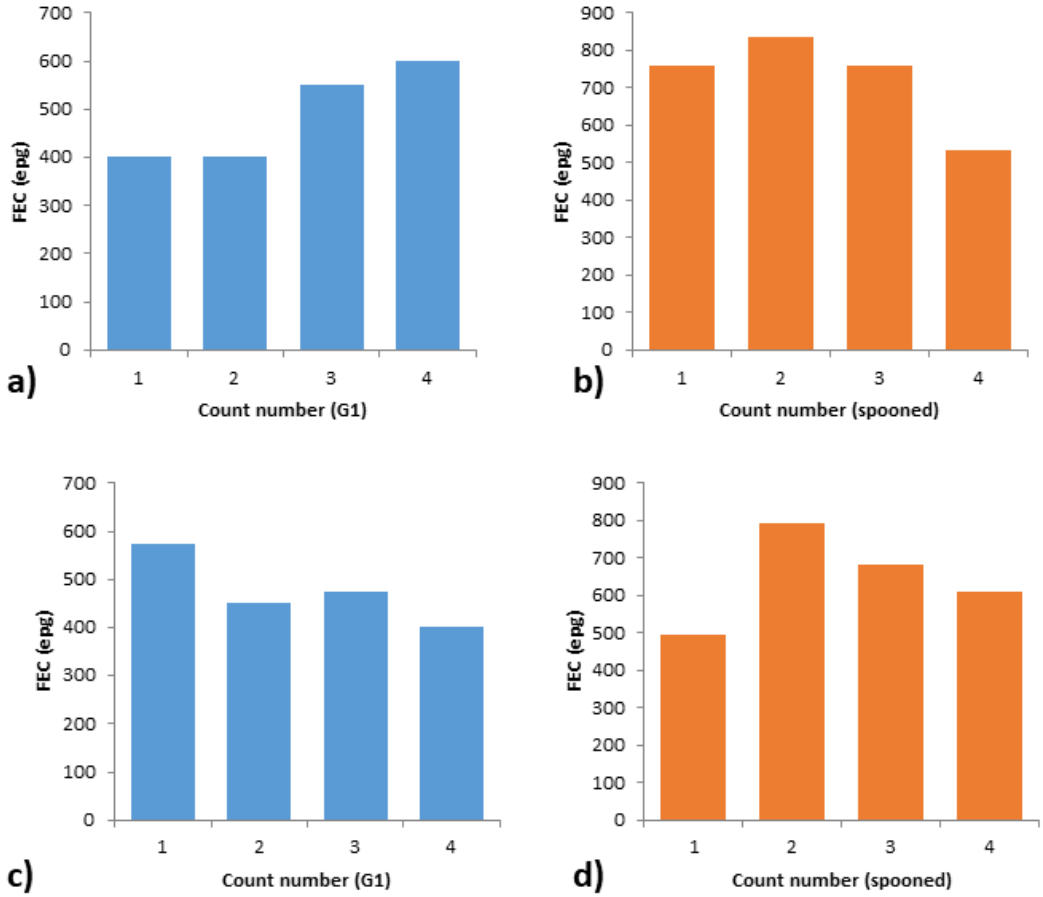

Figure 2-7 a) and b) Replicate 4, and c) and d) Replicate 5. Comparing repeated G1 counts with spooned sub-samples. Separate G1 counts shown (blue bars), compared to single counts from the spooned sub-samples (orange bars) to demonstrate the level of repeatability rather than the counts rising as the sub-sample consistency changed

2.4.4 Slurry fraction investigation - discussion

In order to ensure that the initial sampling of the faecal slurry did not introduce an over or under estimation of eggs into the FEC process the dilution of the faecal material and resulting sampling was assessed. Faecal slurry sampling is of key importance to accurately reflect the distribution of eggs within the slurry of faeces and water which is more challenging as slurry, by its very nature, is a suspension rather than a solution.

Pouring the faecal slurry from the slurry Ziploc bag, demonstrated that the initial watery fraction of the slurry contained fewer eggs than the more solid fraction. A switch from fewer eggs to more eggs is likely due to the eggs being trapped within the solid matter as described in O'Grady and Slocombe (1980). Therefore, as the final FECPAK^{G2} test would involve a single test from each faecal sample, this would always contain the initial watery fraction of the faecal slurry, potentially understating the egg count.

When the slurry was well mixed and repeated subsamples taken by spooning the slurry from the bag rather than pouring it, the counts obtained did not rise with subsequent sub-samples. As nematode eggs are randomly distributed in a faecal sample, some degree of variability is to be expected (Torgerson *et al.*, 2012), however this can be mitigated by thorough mixing of the faecal sample (Morgan e*t al*., 2005). The repeatability of the spooning method was comparable to the repeatability of repeated G1 slides taken from a single preparation.

The concern that a small sample poured from a Ziploc bag would not be representative of the sample as a whole was confirmed. Given the challenges discovered with pouring slurry from a Ziploc bag, an alternative method of

dispensing a small aliquot of slurry was chosen. Aliquoting slurry using a spooning method from the Ziploc bag into the sedimentor rather than pouring it ensured that both watery and solid fractions were sampled. Given the success of the spooning approach this protocol was followed throughout the rest of the optimisation and validation work.

Of note in this initial work, the samples were not sedimented. This was because the purpose of the experiments was solely to determine the distribution of eggs in the slurry, and decide the very first step of the protocol. All further work used a sedimentation step following the initial sub-sampling of the slurry.

2.5 SEDIMENTORS FOR CONCENTRATING EGGS

2.5.1 Concentration of eggs – introduction

A combined sedimentation-flotation technique involves diluting the faecal sample with water and allowing the nematode eggs to gravity sink, before discarding the supernatant and diluting the sediment with a flotation solution. Sedimentation-flotation has been found to be more effective than flotation alone, particularly for cyathostomin eggs (Becker *et al.*, 2016). The SOP for the FECPAK^{G2} test in sheep includes a sedimentation step, and it was decided to assess its usefulness for equine samples also.

Therefore, given the potential importance of sedimentation to improve egg recovery the aim of this section of research was to determine if a greater volume of faecal slurry could be utilised in the sedimentor without the loss of eggs. In doing so, there is an opportunity to concentrate weak samples with a low egg density which would lead to a more sensitive FEC based test.

After a sub-sample of the slurry has been prepared, the next step in the existing FECPAK^{G2} protocol (for sheep) is to dilute the sub-sample with water, and allow it to stand so that the eggs settle to the bottom of the sedimentor. The supernatant containing floating debris is discarded, as well as any lipids that have floated to the top that may subsequently impact on the collection of eggs in the meniscus, and also soluble green pigment that may cloud the final image. The design of the sedimentor traps the bottom 15 ml of liquid, with the eggs and heavier debris, once the supernatant is discarded. When adding 12 ml of slurry to the sedimentor, this ensures that all eggs and debris with a higher specific gravity than water are retained.

2.5.2 Concentration of eggs - materials and methods

The impact of increased faecal slurry volume on sedimentor function was assessed using 50 g of faeces from a horse with a naturally acquired strongyle infection that was mixed with 200 ml water to form the test faecal slurry. The dilution rate of the faecal sample was kept constant with previous work, but a greater total volume of slurry was required to be sufficient for this experiment. A G1 preparation was performed on the slurry according to the manufacturer's instructions (Techion Ltd.) with four slides counted to act as a control.

The faecal slurry was then spooned from the Ziploc bag into four replicate sedimentors. A total of 12 ml, 24 ml, 36 ml and 48 ml of slurry were added to separate sedimentors representing 1×, 2×, 3× and 4× the specified volume of faecal slurry for the sheep procedure (Techion Ltd.). All sedimentors were filled with water and the faecal slurry/water mix was filtered using a 1,000 µm filter and returned to the sedimentor. Each of the sedimentors was allowed to stand for three hours to be confident that all nematode eggs had settled to the bottom of the sedimentor. The supernatant was discarded from each sedimentor and the remaining sediment diluted

with 80 ml of saturated NaCl solution. The sediment/NaCL solution was then filtered in a FECPAK^{G2} cylinder fitted with both 600 µm and 425 µm filters, before being counted using a FECPAK^{G1} slide in triplicate under light microscopy at 40 × magnification.

2.5.3 Concentration of eggs - results.

Following the faecal slurry preparation above, the mean of the triplicate G1 counts, acting as the control, was recorded at 475 epg. It was observed that up to three times the intended volume of slurry could be utilised whilst still producing FECs comparable to the G1 control results (Figure 2-8). The mean FEC for the 12 ml slurry preparation was 520 epg, the mean FEC for the 24 ml slurry was 462 epg and for the 36 ml slurry the mean FEC was 425 epg, which all compared well with the 475 epg control value. At 48 ml slurry preparations the mean FEC dropped to 358 epg.

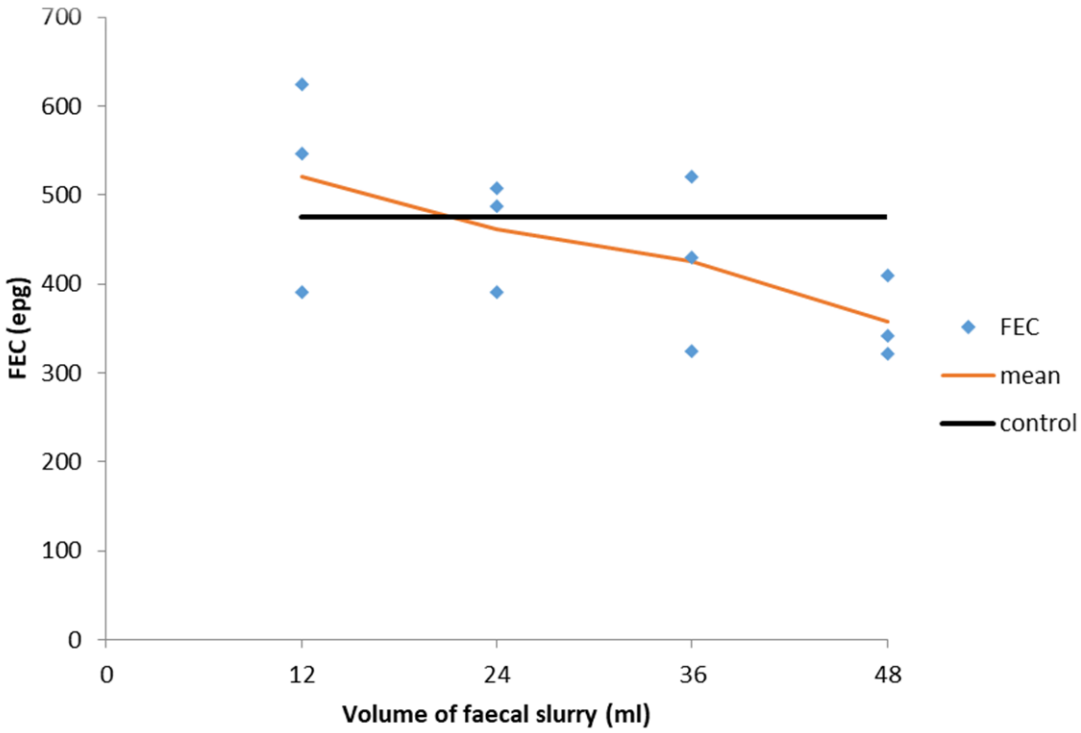

Figure 2-8 FECPAK^{G1} slide counts from sedimented samples, produced using 1×, 2×, 3× and 4× the usual volume of equine faecal slurry. Individual counts shown by blue data points, mean of the triplicate counts shown by the orange line and the control value (mean of three standard un-sedimented G1 counts) shown by the black line.

2.5.4 Concentration of eggs - discussion

The eggs concentrated comparably well up to three times the intended volume of slurry, with 12 ml of slurry giving a mean result of 109% of the control value, 24 ml of slurry giving a mean result of 97% of the control value and 36 ml of slurry giving a mean result of 89% of the control values. Four times the usual volume of slurry (48 ml) gave a mean result of 75% of the control value, suggesting that the limit of concentration using the FECPAK^{G2} sedimentor was around three times the intended sensitivity. It has been found that a combined sedimentation-flotation technique was more effective at identifying nematode-positive samples of many nematode species, particularly cyathostomins (Becker *et al.*, 2016) although at the time of writing it is

believed that this method is not routinely used in quantitative FEC tests other than the FECPAK^{G2}.

From this proof of concept study, it was clear there was potential for a test with a greater level of sensitivity if a higher volume of slurry was exploited. This concept was explored further when the FECPAK^{G2} protocol was adapted to use 20 ml of slurry rather than 12 ml, which would give the FECPAK^{G2,} test a sensitivity of 26 epg rather than 43 epg if using 12 ml slurry. The use of 20 ml slurry would render the sensitivity of the FECPAK^{G2} test similar to that of the FECPAK^{G1}, which stands at 25 epg, and therefore produce a more commercially valuable test.

2.6 VOLUME OF SEDIMENTORS

After the faecal sample has been diluted to make a slurry, the next step in the FECPAK^{G2} methodology is to measure 12 ml into a sedimentor for the sedimentation process. Thus, having ascertained that a more accurate test would be achieved by spooning the slurry into the sedimentor, the next stage was to determine if the sedimentors were an accurate way of measuring a 12 ml aliquot.

The 12 ml slurry line on the sedimentors used for measuring the slurry represents a wide, flat measuring area, and the level itself is marked by a sticker applied to the outside of the sedimentor (Figure 2-9). The aim of this experiment was to determine if the sedimentors were accurately marked at the 12 ml slurry line, as the slurry aliquot was to be measured by spooning in faecal slurry up to this line.

Figure 2-9 The FECPAK^{G2} Sedimentor (Techion Ltd, Dunedin NZ). The 12 ml faecal slurry line is highlighted with an arrow. Also highlighted is side A (circled) for discarding supernatant and side B (boxed) for pouring off the final sample preparation prior to imaging on the Micro-I or slide.

2.6.1 Volume of sedimentors - materials and methods

Nine sedimentors had been supplied for research use, to enable multiple experiments to be performed. Each sedimentor was filled with water up to the 12 ml slurry line, and then the water removed using a serological pipette, quantifying the volume removed from each sedimentor.

2.6.2 Volume of sedimentors - results

In total, the volume of water required to reach the fill line was measured in nine sedimentors. For these nine sedimentors the mean volume of water required to fill was 13.4 ml ± 1.17, with a range of 12 – 16 ml. With the 16 ml sedimentor discarded from analysis (and excluded from further use), the remaining sedimentors had a

mean volume of water required to fill of 13 ml ± 0.7, representing a percentage accuracy of 91% ± 5.9 (Sedimentors measured 109 % ± 5.9 of the 12 ml required).

2.6.3 Volume of sedimentors - discussion

Although not specific to the equine version of the FECPAK^{G2} test, it was considered a useful experiment to determine the accuracy of the sedimentors. The line used to measure the 12 ml of slurry to be added to each sedimentor in the final test was indicated by a sticker applied to the outside of the sedimentor. It was observed that these stickers were not always correctly applied, or had perhaps become dislodged, resulting in some of the sedimentors not being accurately marked – one significantly so. This sedimentor was excluded from further use. Techion was notified of this discrepancy, and it was decided that alternative methods of marking the sedimentors would be investigated, for example printing rather than using stickers.

As section 2.4 "Investigating slurry fractions" had determined that spooning the slurry was the preferred method, consideration was given to the use of a scoop measure instead of measuring using the sedimentors. This would eliminate any variability and potential error due to inaccurate positioning of the sedimentor sticker whilst the optimisation and validation took place.

2.7 SEDIMENTATION TIME TRIAL 12 ML SLURRY

2.7.1 Sedimentation time trial 12 ml - introduction

During the FECPAK^{G2} process, after faecal slurry has been measured into the sedimentor, topped up with water, mixed and then pre-filtered, the G2 protocol requires the sedimentor to stand so that the nematode eggs can sediment which likely

removes any lipids which might impede the imaging process. When first using the FECPAK^{G2} test on lambs, the accuracy compared to the G1 was poor, and Techion Ltd (NZ) postulated that this may have been caused by lipids in the samples (Techion Ltd, personal communication). In addition, little research has been performed on sedimenting nematode eggs and given it has previously been shown that nematode egg recovery is improved with a sedimentation step (Becker *et al*., 2016) it is possible that improved egg recovery due to the sedimentation could be increased.

Therefore, the aim of this set of experiments was to determine the optimal sedimentation time that would collect all of the nematode eggs within the equine faecal slurry sample. By investigating reducing the sedimentation time of nematode eggs during equine FECs there is the potential to yield improvements for the FECPAK^{G2} to perform as quickly as possible without sacrificing accuracy.

2.7.2 Sedimentation time trial 12 ml - materials and methods

The protocol for the original experiment was supplied by Techion NZ, and involved generating a "master mix" of slurry and water in a bucket, and then filling the sedimentors from this bucket. Subsequently, it was decided to run an extra experiment which measured the slurry into individual sedimentors as in the final G2 protocol, in order to ensure that the procedure tested during the optimisation phase was as close as possible to the final test protocol.

Sedimentation time was first assessed using a Techion Ltd. NZ derived protocol. Briefly, a sample of 100 g faeces from a horse with a natural strongyle infection was mixed with 400 ml water to produce a uniform faecal slurry. From this faecal slurry

a G1 preparation was prepared according to the manufacturer's instructions (Techion Ltd. NZ) with three replicate slides counted to produce a control FEC.

A total of 380 ml of the faecal slurry was mixed with 6.27 l of water to replicate the dilution factor used in the sedimentors (normally 12 ml faecal slurry and 198 ml water). From this slurry/water mix, three replicate sedimentors for each time point were filled to the water line. The slurry/water mixture was stirred well between each filling of a sedimentor. The liquid collected in each sedimentor was pre-filtered using a FECPAK^{G2} cylinder fitted with a 1,000 µm filter and returned to the sedimentor which was left to stand for between five and 180 minutes, with an additional sedimentor left to stand overnight. The supernatant was then discarded from each sedimentor and the resulting sediment diluted with 80 ml saturated NaCl and transferred to a FECPAK^{G2} cylinder fitted with 600 µm and 425 µm filters. A FECPAK^{G1} slide was filled from each cylinder and counted using a light microscopy at 40 × magnification.

In addition, sedimentation was also assessed directly following the proposed FECPAK^{G2} methodology. Briefly, a sample of 100 g faeces from a horse with a natural strongyle infection was mixed with 400 ml water to produce a uniform slurry and a G1 preparation was produced in triplicate to act as a control as previously described.

From this faecal slurry, 12 ml was spooned into three replicate sedimentors for each time point. Each sedimentor was filled to the fill line with water. The experiment was then continued as before.

2.7.3 Sedimentation time trial 12 ml - results

In order to assess sedimentation of nematode eggs for equine FECs two methodologies were employed: a Techion Ltd. NZ derived protocol and one reflecting the FECPAK^{G2} protocol. The Techion Ltd. NZ derived protocol demonstrated that a 30 minute sedimentation time was adequate to recover all the eggs (

Figure 2-10) as the FEC at 30 minutes reached the control value and did not fluctuate. In fact, with the exception of an outlier at the 90 minute time point, all the FEC results from 30 minutes on exceeded the FEC from the G1 control value. The sedimented results represent the mean result from the three slides from each time point (one from each cylinder).

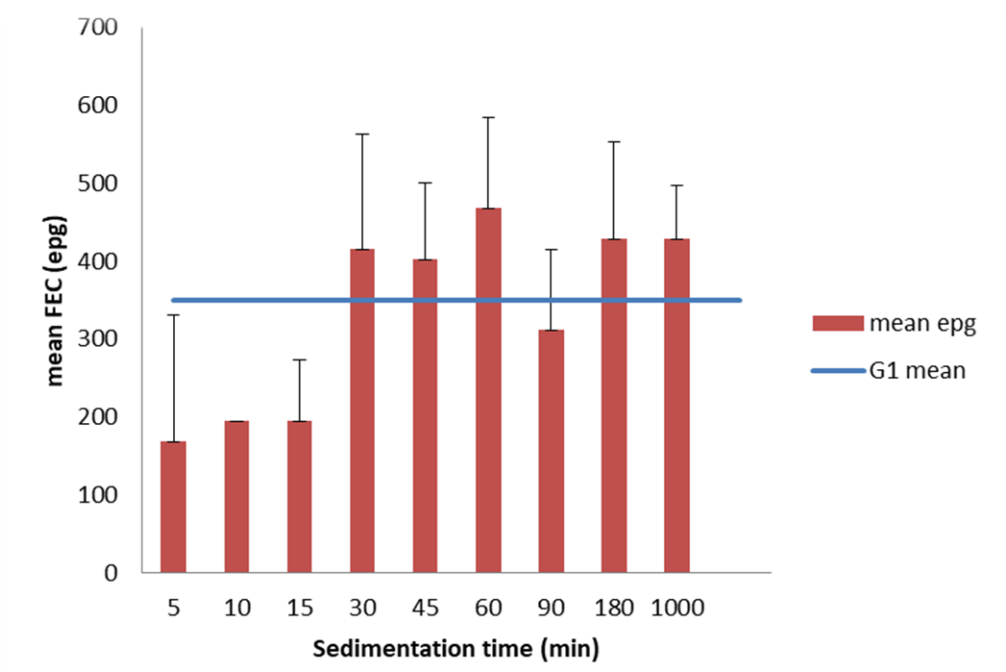

Figure 2-10 Mean FECs for 12 ml protocol with different sedimentation times – Techion protocol. Red bars show the mean of three sedimented counts for each sedimentation time, blue line shows the mean of three G1 counts to act as a control value.

When the faecal slurry was measured using individual sedimentors the average FECs did not reflect the sedimentation pattern of the Techion Ltd. NZ derived protocol (Figure 2-11). The only time point where the mean sedimented FEC reached the control value was after 45 minutes. All additional time points produced FECs lower than the G1 average including fewer eggs recovered at sedimentation times longer than 45 minutes. A line for the mode of the G1 control FECs was included owing to the fact that one of the three counts was much higher than the other two (550 epg compared to 375 epg), and thus may have skewed the results.

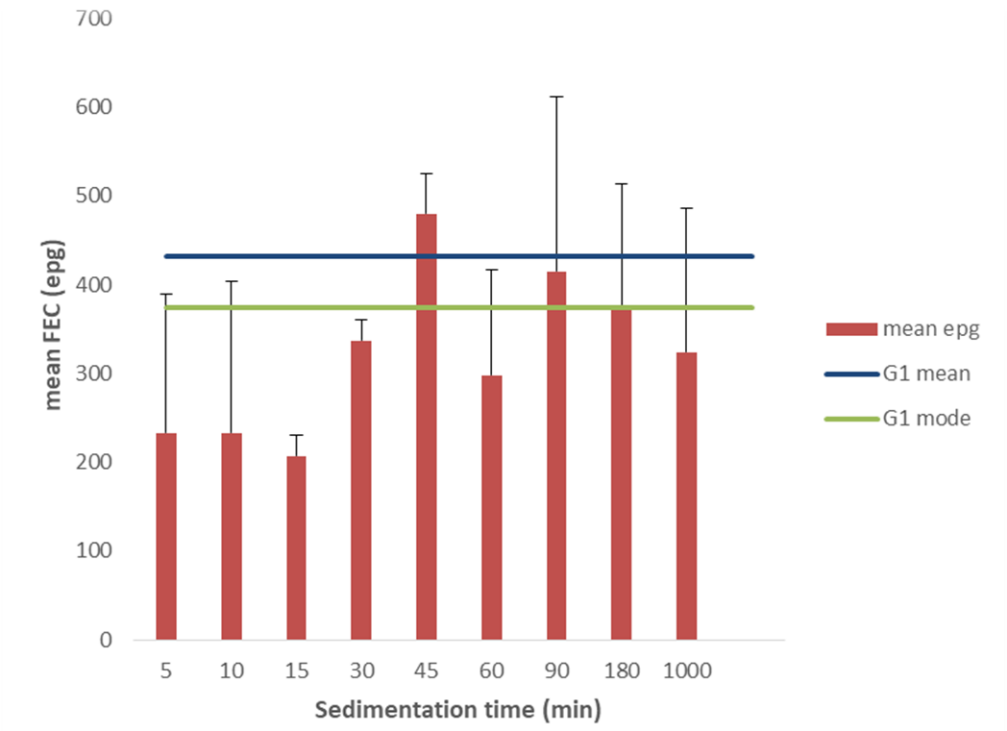

Figure 2-11 Mean FECs for 12 ml protocol with different sedimentation times – measured with sedimentors. Red bars show the mean of three sedimented counts for each sedimentation time, blue line shows the mean of three G1 counts to act as a control value, green line shows the mode of the three G1 counts (included as one of the three was high compared to the other two).

2.7.4 Sedimentation time trial 12 ml - discussion

Optimising sedimentation time for performing equine FECs is potentially of great importance for producing a diagnostic that is both quick and accurate. Therefore, sedimentation of nematode eggs was assessed for equine FECs using two methodologies. When performing the sedimentation time trial according to the protocol from Techion Ltd. NZ, making a faecal master mix prior to filling sedimentors, the results obtained were consistent with the results obtained in the NZ laboratory (Techion NZ Ltd, personal communication), in that a 30 minute sedimentation time was adequate for the majority of eggs to descend to the sediment, in common with other research (Becker *et al.*, 2016). The results from following the FECPAK^{G2} protocol, where individual sedimentors were used to measure the faecal slurry, demonstrated that a 45 minute sedimentation time could be necessary. However, as other literature supported the 30 minute sedimentation time (Becker *et al.*, 2016) and this had been found sufficient in the Techion NZ laboratory for strongyle eggs (Techion NZ, personal communication) the further work was performed using the 30 minutes' sedimentation time. Given the varied results when using the sedimentors to dilute the faecal slurry with saline, the mode of the G1 counts was included. As the three G1 counts were recorded as 550 epg, 375 epg and 375 epg it was likely that the first count may have been unusually high and thus skewed the results. As it happened, only the 45 minute sedimentor showed a FEC (481 epg) as high as the mean G1 control value. Further work on sedimented samples found that a 30 minute sedimentation time was adequate to produce FECs comparable with the control values, so it was likely that this particular experiment was anomalous, rather than showing a trend peculiar to the sedimentor preparation.

2.8 ACCUMULATION TIME TRIAL 12 ML SLURRY

2.8.1 Accumulation time trial 12 ml slurry - introduction

The FECPAK^{G2} cassette, which holds the faecal sample to be imaged by the Micro-I, consists of two wells with a central glass rod through which light passes from underneath (Figure 2-12).

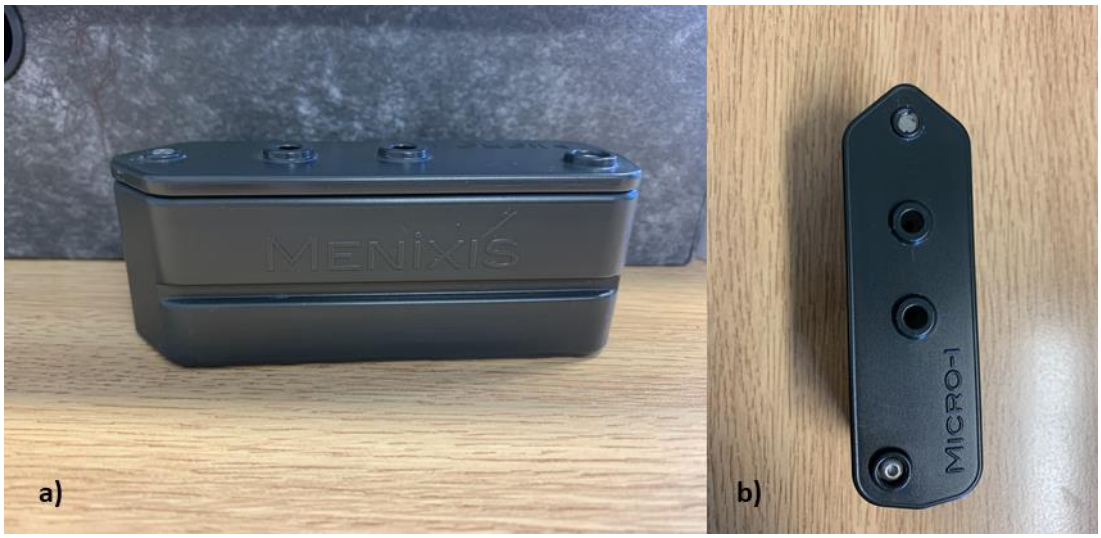

Figure 2-12 Micro-I cassette (Techion Ltd., Dunedin New Zealand) showing a) side view and b) top view.

This process results in a circular illuminated area of 3 mm diameter which forms the final Micro-I captured image. The nematode eggs float to the surface of the saline solution in the cassette, and, due to the domed shape of the meniscus, collect in the central illuminated area (Cooke *et al.*, 2016; Sowerby *et al.*, 2016). This process of nematode eggs floating to the meniscus is termed accumulation. Thus, the accumulation of nematode eggs from equine faecal samples represents an additional area to investigate for suitability for equine FECs. Therefore, the aim of this experiment was to determine the minimal time period required for all nematode eggs to accumulate to the central field of view in the Micro-I cassette.

In addition, one of the equine faecal samples came into the laboratory with an infection of *Parascaris equorum*. In order to capture images of *P. equorum* eggs for Techion Ltd to use in the mark-up training, several cassettes were prepared from this faecal samples, and the opportunity was taken to monitor the accumulation time needed to image *P. equorum* eggs.

2.8.2 Accumulation time trial 12 ml slurry - materials and methods

A sample of 20 g faeces from a horse with a natural strongyle infection was mixed with 80 ml of water to form a uniform faecal slurry. A G1 preparation was produced according to the manufacturer's instructions (Techion Ltd. NZ) counted six times to provide a control value epg. The sample gave variable results, so six replicates were used to form the control value rather than the usual three.

From the faecal slurry, a G2 preparation was performed by spooning 12 ml of faecal slurry into a sedimentor, filling with water and pre-filtering using a 1,000 µm filter before returning the liquid to the sedimentor and allowing it to stand for 30 minutes. The supernatant was discarded, and the sediment mixed with 80 ml of saturated NaCl solution. This liquid was poured into the FECPAK^{G2} cylinder fitted with both 425 µm and 250 µm filters and a 450 µl aliquot used to fill the first well of the imaging cassette.

The FECPAK^{G2} Micro-I software was programmed to capture eight images at 2 minute intervals, imaging immediately and after 2, 4, 6, 8, 10, 12 and 14 minutes. The process of filling the cassette and imaging was repeated in triplicate. All images were examined to determine when all nematode eggs had accumulated to the visible area of the well.

In addition, a sample of 40 g faeces from a one year old horse with a natural strongyle and *P. equorum* infection was mixed with 160 ml water to form a uniform slurry. A G2 preparation was made as above, and used to fill five FECPAK cassettes. The cassettes were allowed to accumulate for 6, 10, 15, 20 and 25 minutes respectively, before being imaged using the Micro-I. A FECPAK^{G1} slide was also filled using the same preparation, and counted to give a control value.

2.8.3 Accumulation time trial 12 ml slurry - results

Accumulation time for nematode eggs from equine faecal samples was investigated in order to optimise the FECPAK^{G2} protocol for equine use. A representative image captured from the Micro-I can be seen in Figure 2-13. The control value, a mean of six G1 counts, was recorded as 179 epg. Following image capture, after altering accumulation times, no further nematode eggs appeared in any replicate after six minutes (Table 2-1). This corresponded to 172 epg for two replicates or 86 epg for the third. In the first replicate, the second egg became out of focus at the ten minute point and therefore disappeared.

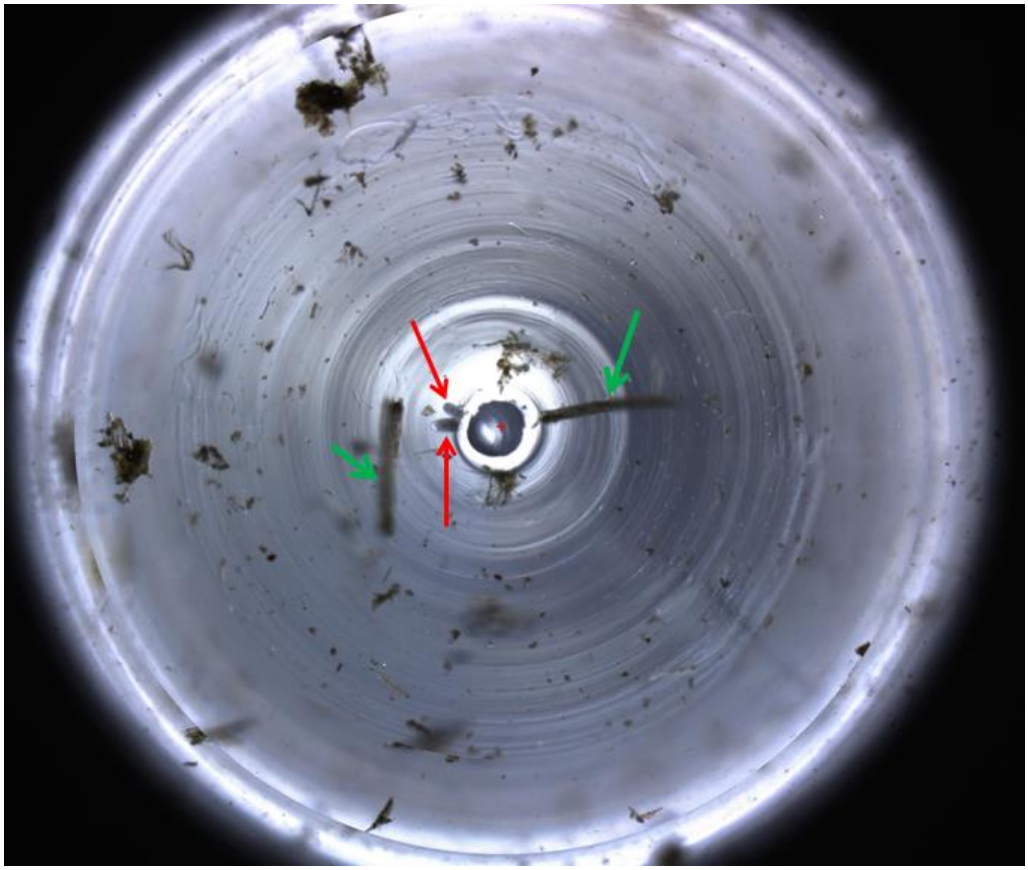

Figure 2-13 Representative image captured from the FECPAK^{G2} Micro-I of an equine faecal sample (Replicate 3 at 6 minutes accumulation). Image represents the central 3 mm of the well, which is the visible field of view. Red cross marks the centre of the image (added by Micro-I software), strongyle eggs marked by red arrows, examples of debris marked by green arrows.

Table 2-1 Accumulation time trial experiment for 12 ml slurry. Repeat images taken at two minute intervals up to 14 minutes, recording the number of eggs that had floated to the centre of the meniscus and into the field of view.

	Replicate 1		**Replicate 2**		**Replicate 3**	
minutes	**Eggs Observed**	**epg**	**Eggs Observed**	**epg**	**Eggs Observed**	**epg**
0	1	86	1	86	1	86
2	1	86	1	86	1	86
4	1	86	1	86	1	86
6	2	172	1	86	2	172
8	2	172	1	86	2	172
10	1	86	1	86	2	172
12	1	86	1	86	2	172
14	1	86	1	86	2	172

The results of the *P. equorum* accumulation experiment are provided in Table 2-2. The same preparation counted on a slide gave a FEC value of 429 epg for strongyle eggs, and 468 epg for *P. equorum*. G2 cassettes allowed to accumulate for ten minutes or longer did appear to image more *P. equorum* eggs than the one with the standard accumulation time of six minutes, although the cassette that accumulated for the longest time did not follow this rule.

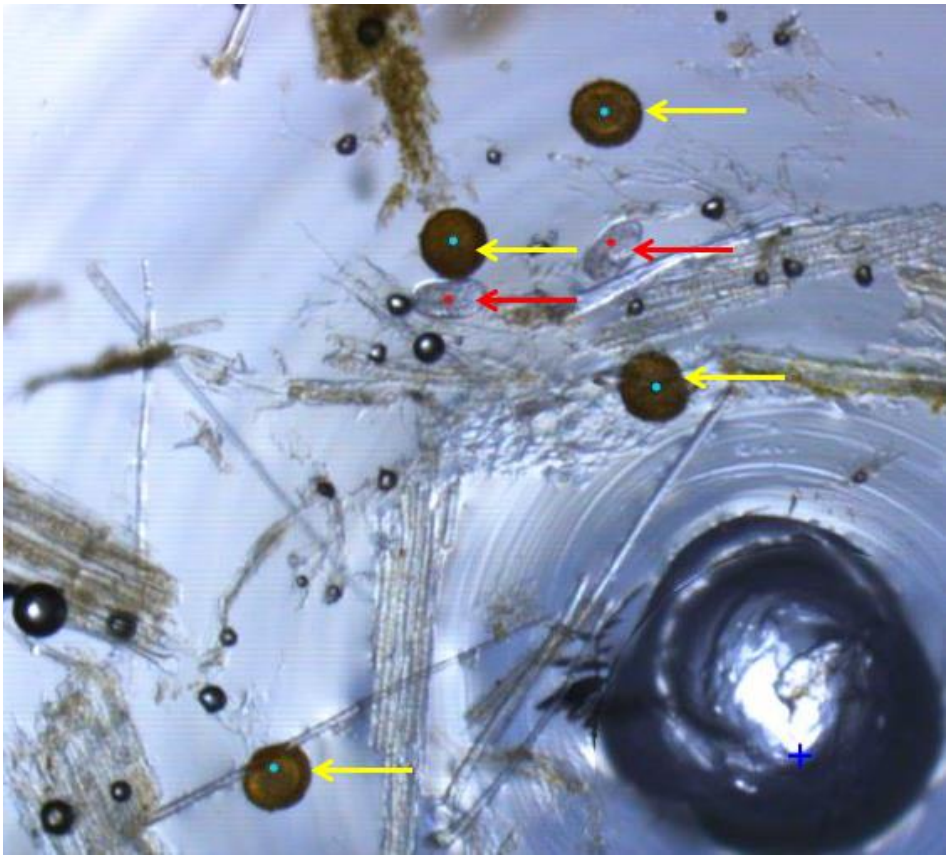

Figure 2-14 Representative image captured from the FECPAK^{G2} Micro-I of an equine faecal sample with a mixed strongyle and *P. equorum* infection. Image represents the central 3 mm of the well, which is the visible field of view. Blue cross marks the centre of the image (added by Micro-I software), strongyle eggs marked by red arrows, *P. equorum* eggs marked by yellow arrows. Blue and red dots added by FECPAK mark-up software.

Table 2-2 Accumulation time trial experiment for *Parascaris equorum*. Five cassettes accumulated for different lengths of time, then imaged using the Micro-I and number of eggs observed recorded.

	Strongyle		***Parascaris equorum***	
minutes	**Eggs Observed**	**epg**	**Eggs Observed**	**epg**
6	14	630	5	225
10	6	270	18	810
15	6	270	12	540
20	4	180	14	630
25	8	360	5	225

2.8.4 Accumulation time trial 12 ml slurry - discussion

In order to ensure as accurate a test as possible, sufficient time has to be allowed for all the nematode eggs within the sample to float to the surface of the solution, and up the curve of the meniscus into the central 3 mm field of view. The possibility existed that an equine faecal sample may not perform in exactly the same way as a ruminant sample. Therefore, the optimum accumulation time was investigated. It appeared that all the eggs that were going to become visible had done so by six minutes. In the first replicate, the second egg that appeared at six minutes lost focus at ten minutes. The Micro-I captures images at different focal planes, and then stacks them together so that the whole surface of the meniscus is in focus. In the second replicate, the image was badly stacked and difficult to read, so this replicate was not a good one to use to draw a conclusion.

The section would likely have been improved if it had been replicated with a greater number of samples, specifically samples with higher egg counts. However, as the optimum accumulation time reached matched with that used for sheep, and also with the results from the New Zealand laboratory, it was considered to be sufficient for the purpose of optimising the preparation of samples for the G2 method. Recent research has demonstrated that eggs which have floated in a flotation solution can sink again (Norris *et al.*, 2019), a point which is worth noting when preparing a number of samples which may unavoidably accumulate for much greater times than six minutes. Potential causes for this sinking effect have been postulated as temperature differences in the flotation solution (Norris *et al.*, 2019), relevant owing to the light source at the base of the Micro-I. It appeared from this preliminary work, that an accumulation time of longer than six minutes might improve *P. equorum* egg accumulation. However, no further samples with a *P. equorum* infection came into

the lab during the optimisation work and so further work to investigate this was not possible. The specific gravity of *P. equorum* eggs is slightly higher at 1.09 compared to 1.05 for strongyle eggs (Norris *et al.*, 2018) and given *P. equorum* eggs are larger than strongyle eggs they must displace a larger amount of fluid in order to float. Recent research confirms that *P. equorum* eggs float more slowly than strongyle eggs, although in the case of both species the speed of flotation would allow them to rise nearly 2 cm in six minutes, double the distance required to accumulate in the centre of the meniscus of the FECPAK^{G2} cassette (Norris *et al.*, 2019). In addition, current recommendation is to treat foals for *P. equorum* as a matter of routine due to the pathogenicity of pre-patent infection (Rendle *et al.*, 2019), so a quantitative diagnosis for these helminths is less important. Nevertheless, *P. equorum* eggs can still be observed in the FECPAK^{G2} test, providing positive diagnosis of infection.

2.9 COMPARING 12 ML SLURRY WITH 20 ML SLURRY

2.9.1 Increasing slurry volume - introduction

Techion highlighted that the sensitivity of the G2, at 43 epg, made it an inferior test to their G1 (sensitivity of 25 epg) and thus were keen to investigate an increased test sensitivity. Earlier work had demonstrated that increased amounts of faecal slurry could be used in the sedimentors without losing eggs, so the potential to match the G1 test sensitivity existed. Thus, a protocol using 20 ml slurry rather than 12 ml was chosen as it that should deliver a sensitivity of 26 epg in the final protocol, similar to that of the G1. Additionally, using the same amount of slurry (12 ml) but using less saline – 50 ml instead of 80 ml was also investigated. Therefore, the aim of this

investigation was to determine if the sensitivity of the test could be increased. This was important because greater sensitivity would mean a more valuable test.

2.9.2 Increasing slurry volume - materials and methods

To investigate different combinations of faecal slurry and saline volumes, a 50 g faecal sample from a horse with a natural strongyle infection was mixed with 200 ml water to make a uniform slurry. A G1 preparation was made from this slurry, and counted on a FECPAK^{G1} slide in triplicate to give a control value. Two sedimentors were prepared with 12 ml slurry, and one with 20 ml slurry, spooned into the sedimentors. Each sedimentor was filled with water, pre-filtered using a 1,000 µm filter, then left to stand for 45 minutes. The supernatant was then discarded and saturated NaCl solution was added to produce one sedimentor with 12 ml slurry and 80 ml saline, one with 12 ml slurry and 50 ml saline, and one with 20 ml slurry and 80 ml saline. These solutions were each added to a FECPAK^{G2}cylinder fitted with 425 µm and 250 µm filters and used to fill three FECPAK^{G1} slides each, which were counted using light microscopy at 40 × magnification.

To compare 12 ml slurry with 20 ml slurry, a 60 g sample of faeces from a horse with a natural strongyle infection was mixed with 240 ml water to make a uniform slurry. A G1 preparation was made according to the usual protocol, apart from the fact that the slurry was spooned into the cylinder instead of being poured. Three FECPAK^{G1} slides were counted to produce a control value. Three sedimentors were prepared with 12 ml slurry, and sedimented for 30 minutes, and three were prepared with 20 ml slurry and sedimented for 45 minutes. All preparations were pre-filtered using 1,000 µm filter prior to sedimentation, then the sediment mixed with 80 ml saturated NaCl, and filtered in the FECPAK cylinder fitted with 425 µm and 250 µm filters. Each preparation was counted in triplicate using the FECPAK^{G1} slide, at a

magnification of 40 ×. A second replicate was performed, with the exception that the standard G1 protocol was followed, pouring the initial faecal slurry rather than spooning it.

To compare different sedimentation times, samples of faeces from two horses with natural strongyle infections were mixed with water (30 g faeces in 120 ml water for sample one, and 50 g faeces in 200 ml water for sample two) to produce a uniform slurry. For each sample a G1 preparation was made (spooned as before). This preparation was counted in triplicate using the FECPAK^{G1} slide at a magnification of 40 ×. Six sedimentors were prepared for each replicate, each using 20 ml slurry and filled with water, then pre-filtered using a 1,000 µm filter. Three sedimentors were left to stand for 30 minutes and three for 45 minutes, then the supernatant was discarded and the sediment mixed with 80 ml saturated NaCl solution. These solutions were filtered in the FECPAK cylinder with 425 µm and 250 µm filters, and counted using the FECPAK slide at 40 × magnification. (NB There was insufficient slurry to fill six sedimentors for sample one, so three were sedimented for 30 minutes and two for 45 minutes).

2.9.3 Increasing slurry volume – results

When comparing different combinations of faecal slurry volume and saline volume, all the combinations produced mean FECs of above the G1 control value. The G1 mean control FEC was 293 epg, the mean of the 12 ml slurry / 80 ml saline counts was 442 epg, the mean of the 12 ml slurry / 50 ml saline was 396 epg and the mean of the 20 ml slurry / 80 ml saline combination was 383 epg (Figure 2-15). Paired sample t-tests demonstrated that the mean of each combination was not significantly different to the mean of the G1 control value in any case (two tailed $p > 0.05$).

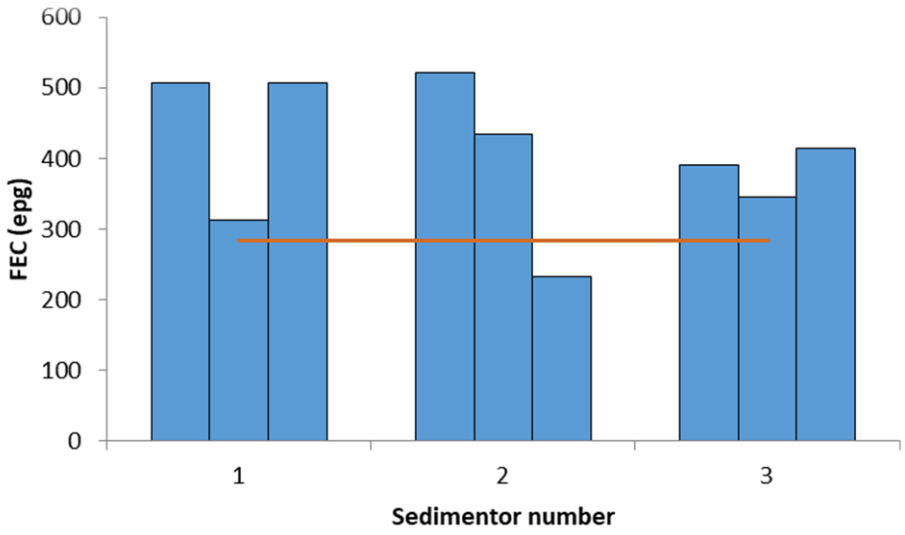

Figure 2-15 Comparing different volumes of slurry and saline – Slurry:Saline (ml:ml) ratios for each sedimentor were as follows; Sedimentor 1- 12:80, Sedimentor 2 - 12:50 , Sedimentor 3 - 20:80. G1 control mean shown by orange line.

When comparing 12 ml slurry sedimented for 30 minutes with 20 ml slurry sedimented for 45 minutes, two replicates were performed. The mean of the triplicate spooned G1 counts was 300 epg, the mean of the 12 ml preparation sedimented for 30 minutes was 286 epg and the mean of the 20 ml preparation sedimented for 45 minutes was 233 epg (Figure 2-16 a). The mean of the triplicate poured G1 counts was 217 epg, the mean of the 12 ml preparation sedimented for 30 minutes was 327 epg and the mean of the 20 ml preparation sedimented for 45 minutes was 299 epg (Figure 2-16 b). Paired samples t-tests showed that the mean of the G1 counts was not significantly different to the sedimented preparations in either case (two tailed $p > 0.05$), and that in neither case was the mean of the 12 ml preparation significantly different to the mean of the 20 ml preparation (two tailed $p > 0.05$).

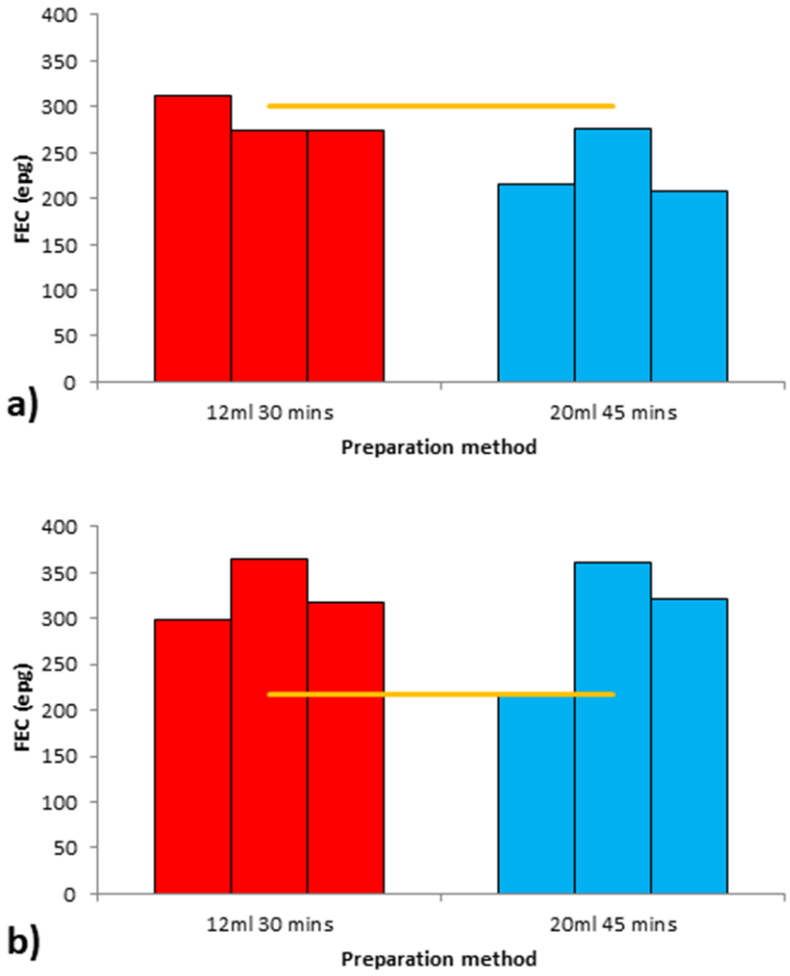

Figure 2-16 FECs produced from 12 ml slurry and 80 ml saline when sedimented for 30 minutes (red bars) or 20 ml slurry and 80 ml saline when sedimented for 45 minutes (blue bars). G1 control mean shown by yellow line. a) First replicate with G1 controls produced by spooning the faecal slurry, b) second replicate with G1 controls prepared in the standard manner (pouring the faecal slurry).

To compare sedimentation times, two replicates were prepared using 20 ml of faecal slurry and 80 ml of saline, with three sedimentors left for 30 minutes and three for 45 minutes (only two were left for 45 minutes in replicate one due to insufficient faecal slurry available). There was no clear benefit in sedimenting for 45 minutes. Mean G1 control value in the first replicate was 250 epg, with 30 minutes sedimentation giving a mean of 253 epg and 45 minutes sedimentation a mean of 288 epg. Mean G1 control value in the second replicate was 267 epg, with 30 minutes sedimentation giving a mean of 258 epg and 45 minutes sedimentation a mean of 238 epg (Figure 2-17). Paired sample t-tests on the data from the second replicate showed no significant differences between the mean FECs from the 30 minute sedimentation or the 45 minute sedimentation, and neither was significantly different from the mean G1 control (two-tailed $p > 0.05$). Paired sample t-tests could not be performed on the data from the first replicate because only two data points for 45 minutes' sedimentation were available.

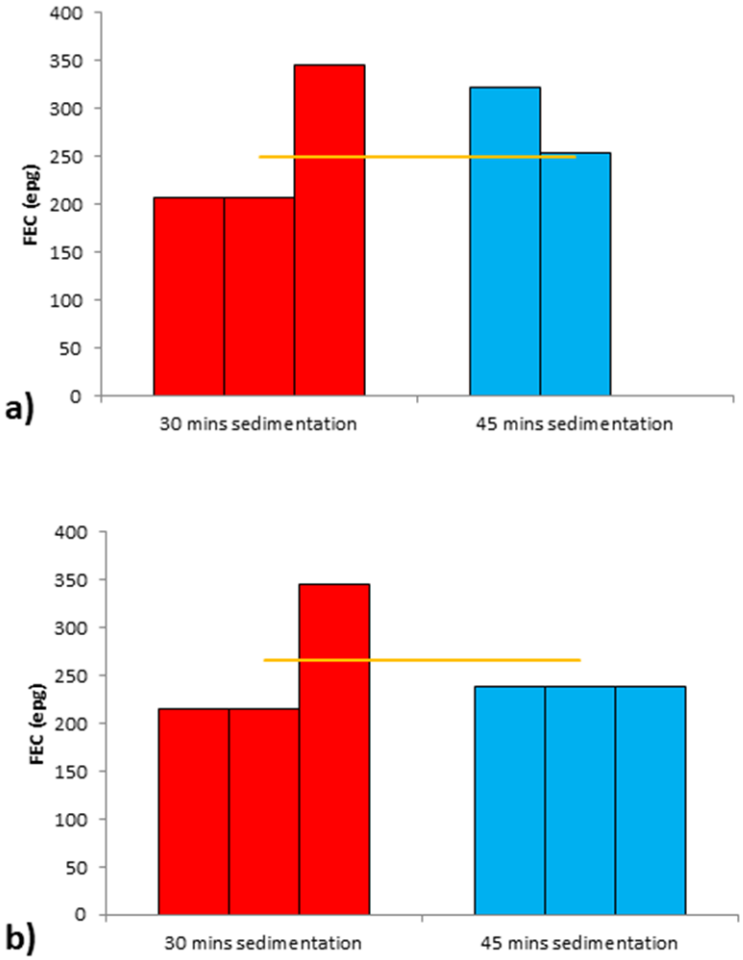

Figure 2-17 FECs produced from 20 ml slurry and 80 ml saline when sedimented for 30 minutes (red bars) or 45 minutes (blue bars). G1 control mean shown by yellow line. a) and b) represent two replicates (NB there was insufficient faecal slurry to prepare the sixth sedimentor for the first replicate)

2.9.4 Increasing slurry volume - discussion

When comparing different combinations of faecal slurry volume and saline volume, all the combinations produced mean FECs of above the G1 control value. However, following the results, the 20 ml slurry and 80 ml saline protocol was chosen to investigate further given it gave greater sensitivity than either 12 ml / 80 ml or 12 ml / 50 ml. In addition, it was easier to work with a greater amount of liquid, as the 12 ml / 50 ml protocol did not produce sufficient sample to be easily pipetted from the FECPAK^{G2} cylinder. The 12 ml slurry and 50 ml saline was not investigated further for these reasons.

Following selection, the original 12 ml protocol was compared with the improved sensitivity 20 ml protocol, which demonstrated that the 20 ml protocol produced comparable results to the 12 ml protocol. It had been decided to spoon the slurry into the G1 cylinder in order to be consistent with the G2 protocol. This spooned G1 protocol produced higher FECs compared to the G2 preparations from the same slurry. When this was mentioned to Techion, the advice was to discontinue this practice, as the G1 method had been validated using the pouring method, so this should be regarded as the correct protocol. In the first replicate, because the G1 protocol had been changed, the results were poorer than the G1 control results. When a second replicate was performed following the original G1 protocol, the 20 ml protocol produced higher FECs than the control results, and it was decided to investigate the 20 ml protocol further.

It was proposed that a greater amount of faecal slurry used might need a longer sedimentation time, so initial work compared a 30 minute sedimentation time with a 45 minute sedimentation time. In the first replicate, the first two sedimentors gave higher FECs after 45 minutes sedimentation. However, the initial slurry did not

provide sufficient volume to prepare three sedimentors for each sedimentation time period, so a second replicate was performed. This replicate had three sedimentors from each preparation counted in order to produce mean values. As FECs naturally vary between counts taken from the same horse, mean values would help smooth out these variations and better inform which protocol was preferable. Initially, it was thought that it might have been necessary to sediment a 20 ml sample for longer than the 12 ml samples (45 minutes instead of 30 minutes), but this initial work did not demonstrate a consistent increase in FEC following 45 minutes sedimentation. Paired sample t-tests showed that there was no significant difference between the different slurry volumes or the different sedimentation times (two-tailed $p>0.05$), meaning that any differences in the mean counts observed could be explained by chance variation in the FECs obtained.

This initial work comparing the original 12 ml G2 protocol with a more sensitive 20 ml G2 protocol demonstrated the potential to increase the sensitivity of the G2 test by using a greater amount of faecal slurry. It was decided to repeat the sedimentation and accumulation time trials using the new 20 ml protocol.

2.10 SEDIMENTATION TIME TRIAL 20 ML FAECAL SLURRY

2.10.1 Sedimentation time trial 20 ml - introduction

Having previously ascertained that the potential existed to develop a more sensitive G2 test, the measuring of optimal sedimentation time was repeated using the new 20 ml protocol. The aim of this set of experiments was to determine the minimal sedimentation time that would trap all of the eggs in a 20 ml faecal slurry sample.

2.10.2 Sedimentation time trial 20 ml - materials and methods

This work was an exact replicate of the experiments performed in Section 2.7 Sedimentation time trial 12 ml slurry, except that in the first experiment 700 ml of the faecal slurry was mixed with 6.65 litres of water to replicate the dilution rate in the sedimentors when 20 ml of slurry and 190 ml water are used. In the second experiment, each sedimentor was prepared by spooning in 20 ml of faecal slurry and filling to the fill line with water and, to save time, only time points of 5, 30, 45 and 1,000 minutes were tested. In both of the two experiments, the 425 µm and 250 µm filters were used in the FECPAK^{G2} cylinder.

2.10.3 Sedimentation time trial 20 ml - results

Two methodologies were again used to assess the length of time needed to sediment faecal samples for the 20 ml G2 FEC protocol. The Techion Ltd. NZ protocol demonstrated that a 30 minute sedimentation time was sufficient for all eggs to be recovered, as the FEC at 30 minutes reached the G1 control value and did not continue to increase with further sedimentation time (Figure 2-18). The sedimented results represent the mean result from the three slides from each time point (one from each cylinder).

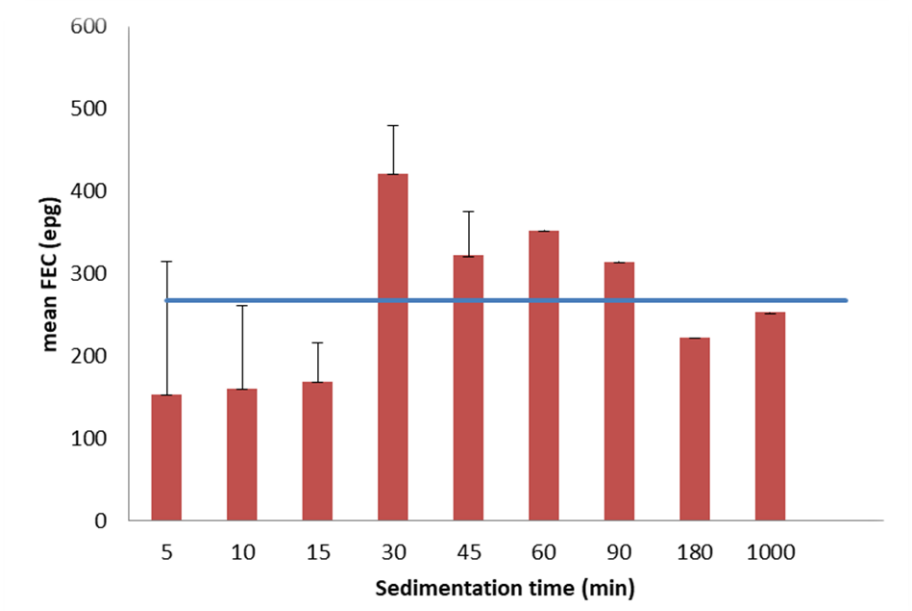

Figure 2-18 Mean epg for 20 ml protocol with different sedimentation times – Techion protocol. Red bars show the mean of three G2 counts for each sedimentation time, the blue line shows the mean of three G1 counts to act as a control value.

When the faecal slurry was measured using individual sedimentors, no advantage was observed from sedimenting for 45 minutes over 30 minutes, although more eggs were recovered by the sedimentors left overnight (Figure 2-19).

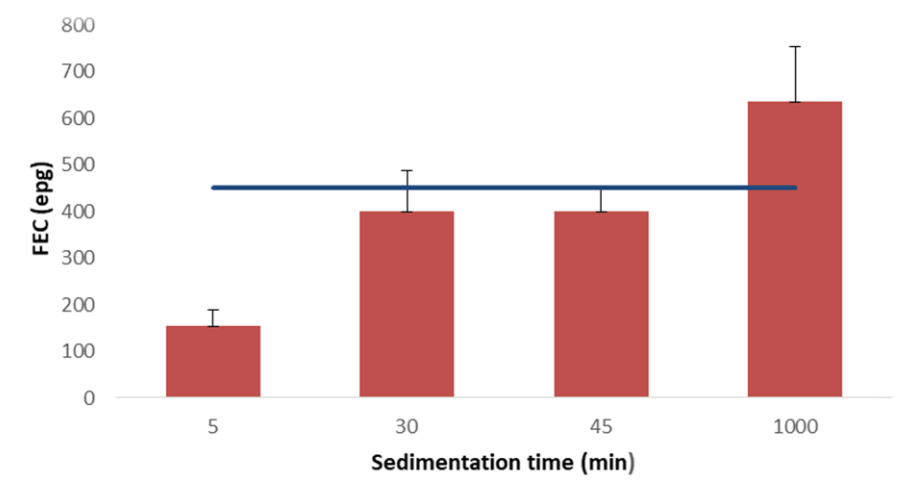

Figure 2-19 Mean FEC for 20 ml protocol with different sedimentation times – measured with sedimentors. Red bars show the mean of three sedimented counts for each sedimentation time, the blue line shows the mean of three G1 counts to act as a control value.

2.10.4 Sedimentation time trial 20 ml - discussion

This work confirmed that a thirty minute sedimentation time was still sufficient and that the time did require an increase to 45 minutes for the 20 ml protocol. It was interesting to note that the results from FECs measured using the 20 ml scoop into the sedimentors produced lower FECs than the control figures, whereas measuring using a "master mix" in a bucket produced higher FECs than the control figures. However, it is possible that the control figures were where the difference occurred. The mean G1 count for the bucket experiment was 267 epg and the mean G1 count for the sedimentor measured experiment was 450 epg, despite the samples having come from the same horse only four days apart. The mean value for the sedimented samples after 30 minutes' sedimentation time was 422 epg for the bucket measure, and 399 epg for the sedimentor measure, so the sedimented values were more consistent with each other than was the case with the control values.

2.11 ACCUMULATION TIME TRIAL 20ML SLURRY

2.11.1 Accumulation time trial 20 ml – introduction

Therefore, having confirmed that the potential existed to develop a more sensitive G2 test, the previous work determining optimal accumulation time was repeated using the new 20 ml protocol. The aim of this new set of experiments was to determine the shortest accumulation time required for all nematode eggs in a 20 ml faecal slurry sample to accumulate in the central field of view in the Micro-I cassette.

2.11.2 Accumulation time trial 20 ml – materials and methods

This work was an exact replicate of the work performed in section 2.8 Accumulation time trial 12 ml slurry except that the initial faecal slurry was prepared from a

sample of 40 g faeces from a horse with a natural strongyle infection was mixed with 160 ml of water, to give a larger volume of faecal slurry to work with. A G1 preparation was made and three slides counted to give a control value.

From the slurry, a G2 preparation was made by spooning 20 ml of slurry into a sedimentor, and then the experiment continued as described in section 2.8 Accumulation time trial 12 ml slurry.

2.11.3 Accumulation time trial 20 ml – results

Accumulation time for nematode eggs from faecal samples prepared with 20 ml of faecal slurry was investigated in order to optimise the FECPAK^{G2} protocol for equine use. The control value, a mean of three G1 counts as recorded as 400 epg. All eggs that were going to appear were visible by the time four minutes had passed (two minutes in the case of replicate three), as shown in Table 2-3. This corresponded to 208 epg for replicate one and 468 epg for replicate three. Replicate two produced images with bad stacking images, which could not be read. At the 12 minute time point, a stacking issue also obscured one of the previously visible eggs in replicate one.

Table 2-3 Accumulation time experiment, 20 ml slurry. Repeat images taken at two minute intervals up to 14 minutes, recording the number of eggs that that had floated to the centre of the meniscus and into the field of view. NB The images on replicate 2 had bad stacking issues, and could not be read. NB O/F = Out of Focus, 3* = 3 eggs visible and one obscured

	Replicate 1		**Replicate 2**		**Replicate 3**	
minutes	**Eggs Observed**	**epg**	**Eggs Observed**	**epg**	**Eggs Observed**	**epg**
0	1	52	0	0	0	0
2	O/F	O/F	0	0	9	468
4	4	208	0	0	9	468
6	4	208	0	0	9	468
8	4	208	0	0	9	468
10	4	208	0	0	9	468
12	3 *	156	0	0	9	468
14	4	208	0	0	9	468

2.11.4 Accumulation time trial 20 ml – discussion

In order to ensure as accurate a test as possible, sufficient time has to be allowed for all the nematode eggs within the sample to float to the surface of the solution, up the curve of the meniscus and into the central 3 mm field of view. The possibility existed that the preparation made using 20 ml of faecal slurry might behave differently to that made with 12 ml slurry, and thus the experiment was repeated.

Although the images produced in this experiment were not of very good quality, the results did not suggest that an accumulation time of longer than six minutes would be required with the 20 ml protocol, and so this figure was retained. The software supplied with the Micro-I to customers (as opposed to that supplied for laboratory use to test the machine) includes an automatic delay to allow eggs to accumulate. Because this delay is set at six minutes for sheep samples, it was decided that this would be retained for the horse protocol, as it was more than sufficient for all eggs to accumulate.

2.12 COMPARING FILTER SIZES AND PREPARATION METHODS

2.12.1 Preparation methods - introduction

Part way through the optimisation, a new set of filters were supplied by Techion Ltd. NZ, (referred to in the text as the black filters), for insertion into the FECPAK^{G2} cylinder. These were 425 µm and 250 µm, replacing the silver filters that had previously been supplied that were 600 µm and 425 µm. The finer black filters had been produced for use in the FECPAK^{G2} test for human samples, and it was necessary to determine which filters would be most suited to the FECPAK^{G2} system for equine use. It was decided to compare the results with the different slurry volumes and with the different filter sizes to determine which protocol worked optimally when FEC samples were imaged using the Micro-I and cassette.

Therefore, the aim of this investigation was to ensure that the filter size and slurry volume selected for the final protocol also performed well when imaged using the Micro-I and cassette. This was important in order that any vagaries caused by the

imaging process were accounted for, in case a preparation which performed well when viewed under a slide did not perform well when imaged using the cassette.

2.12.2 Preparation methods - materials and methods

A sample of 80 g faeces from a horse with a natural strongyle infection was mixed with 320 ml water to give a uniform slurry. A G1 preparation was made, and three slides counted to give a control value.

The filters used in this experiment were: White = 1,000 µm (used consistently as a pre-filter), Silver = 600 µm and 425 µm and Black = 425 µm and 250 µm. Six sedimentors were prepared using this same slurry, by pre-filtering either once or twice, sedimenting for 30 minutes, then adding 80 ml of saturated NaCl to the sediment and filtering through either the black or the silver filters. Details of the filter sizes and slurry volumes used for each sedimentor are contained in

Table 2-4.

Table 2-4 Slurry volumes and filter sizes used, giving the size of the first and second pre-filter (if two pre-filtration steps used), and size of final filters in FECPAK^{G2} cylinder.

Sedimentor	Slurry volume	First pre-filter	Second pre-filter	Filters for final solution
1	20 ml	White	-	Black
2	20 ml	White	-	Silver
3	12 ml	White	-	Black
4	12 ml	White	-	Silver
5	20 ml	White	Silver	Black
6	12 ml	White	Silver	Black

Each preparation was used to fill two FECPAK^{G2} cassettes and counted using the Micro-I. Preparation five was used to fill three cassettes, because the second cassette produced few eggs.

2.12.3 Preparation methods - results

Six preparations were produced with differing amounts of faecal slurry, filtered with the different sizes of filter. Preparations one to six were used to fill two FECPAK^{G2} cassettes each, with the exception of preparation five mentioned above, which was used to fill three cassettes. The results given are the mean of the counts for each preparation.

No clear difference was observed between any of the methods performed (Figure 2-20). It appeared that the 20 ml preparation performed to a higher standard with the black filters whilst the 12 ml preparation performed optimally with the silver filters. Furthermore, introducing a second pre-filter step did not provide consistently better results over the two different faecal slurry volumes.

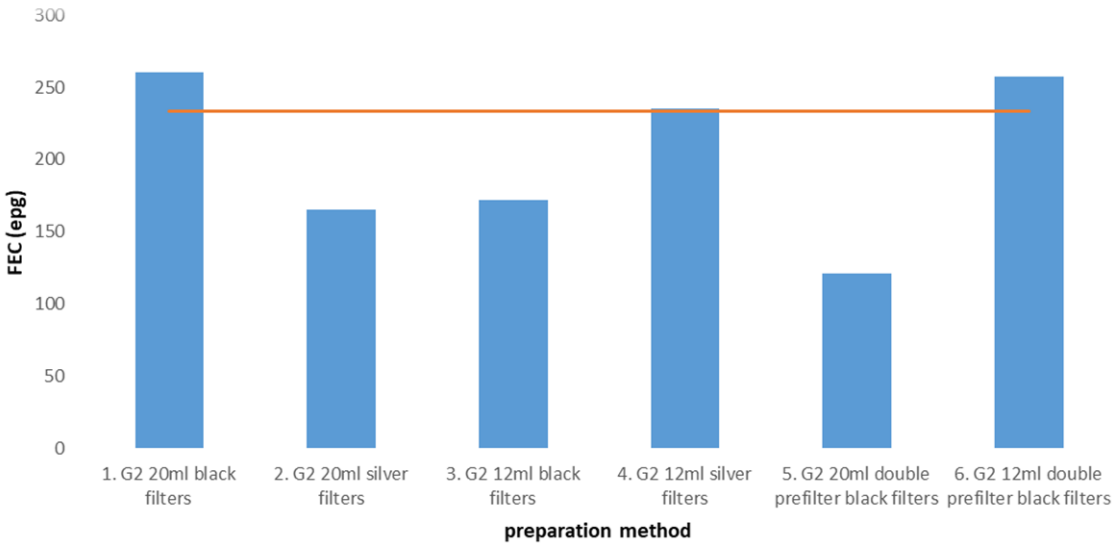

Figure 2-20 Different slurry volumes and filtration steps, imaged using the G2 cassette and Micro-I. Mean G2 cassette FEC values for each preparation method shown by blue bars, G1 control mean shown by orange line.

2.12.4 Preparation methods - discussion

Filtering the faecal slurry is an important step in the FECPAK^{G2} sample preparation, as the process concentrates both nematode eggs and floating debris in a 3 mm field of view for imaging. If too much floating debris is present, there is the possibility of it obscuring the eggs, so a much clearer image is required for imaging in the cassette and Micro-I than would be required to count the preparation using a slide. Thus, this experiment was designed to see if there were any easily visible differences between the different filtration methods. However, the result was inconclusive as no filtration method performed consistently better with both slurry volumes. When analysing the captured images from the cassette, all of them suffered from a wetting issue, defined as the liquid failing to adhere to the light rod, and thus there was no clear difference between either the amount of debris on the images or the accumulation of the eggs to the centre of the field of view with each filter size. This issue was discussed with Techion Ltd, who suggested soaking the light rod in Virkon disinfectant (Dupont Ltd) which cured the wetting issue in subsequent imaging work. More work was required in order to determine separately the best slurry volume, and then the best filter size to use with the selected volume, described in Sections 2.13 and 2.14.

2.13 COMPARING 12 ML VS 20 ML USING CASSETTE

2.13.1 Comparing slurry volume in cassette – introduction

As there was no easily visible difference between the different preparation methods in the experiment described in Section 2.12 above, it was decided to treat the two variables (slurry volume and filter size) separately. Firstly, the slurry volumes were

compared and imaged using the Micro-I to determine which protocol was better for the full test – the more sensitive 20 ml protocol, or the less sensitive 12 ml one.

Thus, the aim of these experiments was to determine which slurry volume performed best when imaged using the cassette and Micro-I. This was important in order to ensure that the test was as sensitive as possible, whilst maintaining accuracy.

2.13.2 Comparing slurry volume in cassette - materials and methods

A sample of 60 g of faeces from a horse with a natural strongyle infection was mixed with 240 ml water to produce a uniform slurry. Two G1 preparations were made, the first was counted three times and the second counted once. These values were used to produce a mean value as a control, with the second G1 count taken so that the results could potentially be used as part of the validation, depending on which protocol was selected as the final method.

Four sedimentors were prepared, two with 12 ml slurry and two with 20 ml slurry. These were pre-filtered using a 1,000 µm filter, then left to stand for 30 minutes. The supernatant was discarded, and 80 ml saturated NaCl was added to the sediment. This liquid was filtered using the FECPAK^{G2} cylinder fitted with 425 µm and 250 µm (black) filters, and two FECPAK^{G2} cassettes filled from each preparation and imaged using the Micro-I.

Two more replicates were performed, using faecal samples from different horses. A further four replicates were also processed in the same way, aside from the fact that the FECPAK^{G2} cylinder was fitted with the 600 µm and 425 µm (silver) filters.

2.13.3 Comparing slurry volume in cassette - results

Each replicate of the different slurry volumes was imaged using the cassette and Micro-I and Figure 2-21a provides the epg returned from each combination of sample and method (height on the y axis of the boxes) and also the repeatability for each method with each sample (the size of each box plus whisker).

Height of the boxes on the y axis in Figure 2-21b demonstrates the epg figures from all samples for each method; size of the boxes plus whiskers shows the repeatability of each method across all samples.

Both methods of comparing the data (Figure 2-21) demonstrated that the 12 ml protocol returned a higher egg count than the 20 ml protocol in the majority of the samples. The mean of the counts from the 20 ml protocol represented 77% of the G1 control values, whereas the mean of the counts from the 12 ml protocol were 105% of the G1 control values. However paired sample t-tests on the mean count for each method showed no significant difference between the G1 and either the 12 ml or the 20 ml FECs, or between the 12 ml FECs and the 20 ml FEC (two-tailed $p > 0.05$).

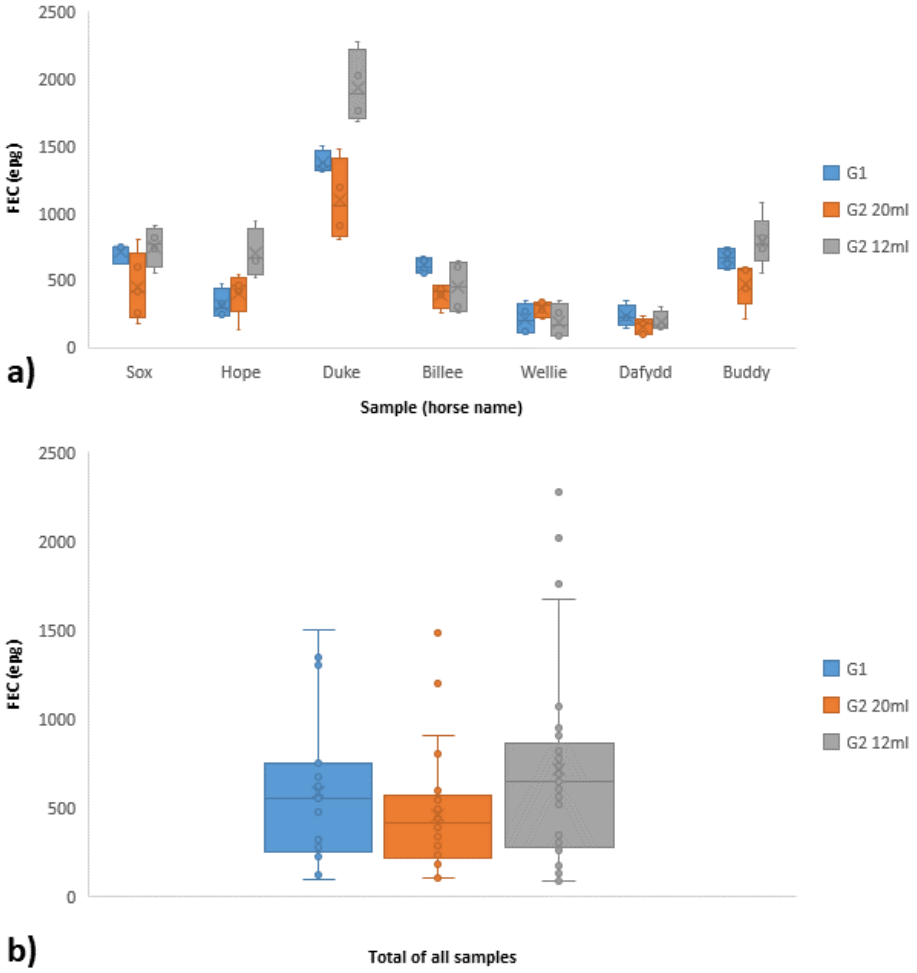

Figure 2-21 Comparing slurry volumes, imaged using the G2 cassette and Micro-I a) Data comprises each G2 count: epg returned from each combination of sample and method (height on the y axis of the boxes) and also the repeatability for each method with each sample (the size of each box plus whisker) b) Data comprises the total counts for all samples: epg returned from each method (height on the y axis of the boxes) and also the repeatability for each method (the size of each box plus whisker)

2.13.4 Comparing slurry volume in cassette - discussion

Thus, increasing the sensitivity of the FECPAK^{G2} test would clearly deliver a more valuable assessment when performing a faecal egg count reduction test (FECRT) as is required to measure anthelmintic resistance. FECRT involves testing both before and after treatment and calculating the percentage reduction in FEC (Kaplan, 2002), and can only be achieved at low infection levels by using sensitive test

methodologies (Coles, 2009; Levecke *et al.*, 2011). Initial work comparing the more sensitive 20 ml protocol with the original 12 ml protocol using the G1 slide to perform the counts demonstrated that the two protocols were similar in their ability to recover nematode eggs. However, despite not being statistically significant, it was clear from this work that when imaging these preparations using the cassette and Micro-I, there was a trend for the less sensitive 12 ml protocol to produce higher egg counts than the 20 ml protocol. On average, the 12 ml protocol also gave results that were more consistent with the G1 control results, with a mean accuracy level of 105% of the G1 control values, compared to 77% in the case of the 20 ml protocol. Given these results, the decreased sensitivity of the 12 ml protocol was outweighed by a greater accuracy when imaging using the G2 cassette and Micro-I, although the 20 ml protocol counted on a FECPAK^{G1} slide was utilised in later work on anthelmintic resistance monitoring.

2.14 COMPARING STANDARD SILVER AND FINER BLACK FILTERS

2.14.1 Filter sizes - introduction

As previously mentioned, the FECPAK^{G2} cassette concentrates the nematode eggs and floating debris into a small 3 mm diameter field of view in order to produce small images that can be transmitted via the internet for diagnosis by specialist technicians. This requires as much of the floating debris as possible to be filtered out, whilst retaining all the nematode eggs in the sample. Previous work had failed to determine whether there was an improvement using the finer, black filters. After having decided on the 12 ml protocol as the optimal version for imaging using the cassette, it was then necessary to determine if the black or the silver filters produced

higher quality images. Therefore, the aim of this section was to determine the optimum filter size for the selected 12 ml protocol.

2.14.2 Filter sizes - materials and methods

A sample of 60 g of faeces from a horse with a natural strongyle infection was mixed with 240 ml water to produce a uniform slurry. Two G1 preparations were made from this slurry, the first being counted in triplicate and the second counted once, using the FECPAK^{G1} slide. These values were used to produce a mean value as the control. The second G1 preparation was included so that the results could be used as part of the validation of the final protocol.

Four sedimentors were prepared by pouring 12 ml slurry, pre-filtering using the 1,000 µm filter and sedimenting for 30 minutes. The supernatant was discarded, 80 ml saturated NaCl added to the sediment and then the liquid added to a FECPAK cylinder. Two of the cylinders were fitted with the 600 µm and 425 µm (silver) filters and two with the 425 µm and 250 µm (black) filters. Two cassettes from each cylinder were imaged using the Micro-I. Seven replicates were performed, using faeces from different horses.

2.14.3 Filter sizes - results

FECPAK^{G2} preparations were made using the selected 12 ml faecal slurry protocol, and filtered using either the standard silver filters or the finer black filters. The FECs obtained are given in Figure 2-22. Height on the y axis of each box plus whisker in Figure 2-22a shows the epg figures returned for each sample with each filter size, and size of the boxes plus whiskers shows the repeatability for each sample and filter size combination. Height of the boxes on the y axis in Figure 2-22b shows the epg figures from all samples for each filter size, size of the boxes plus whiskers shows

the repeatability of each filter size across all samples. The mean of the FECs obtained by the G2 counts was 99% of the G1 controls in the case of the black filters, and 102% of the G1 controls in the case of the silver filters.

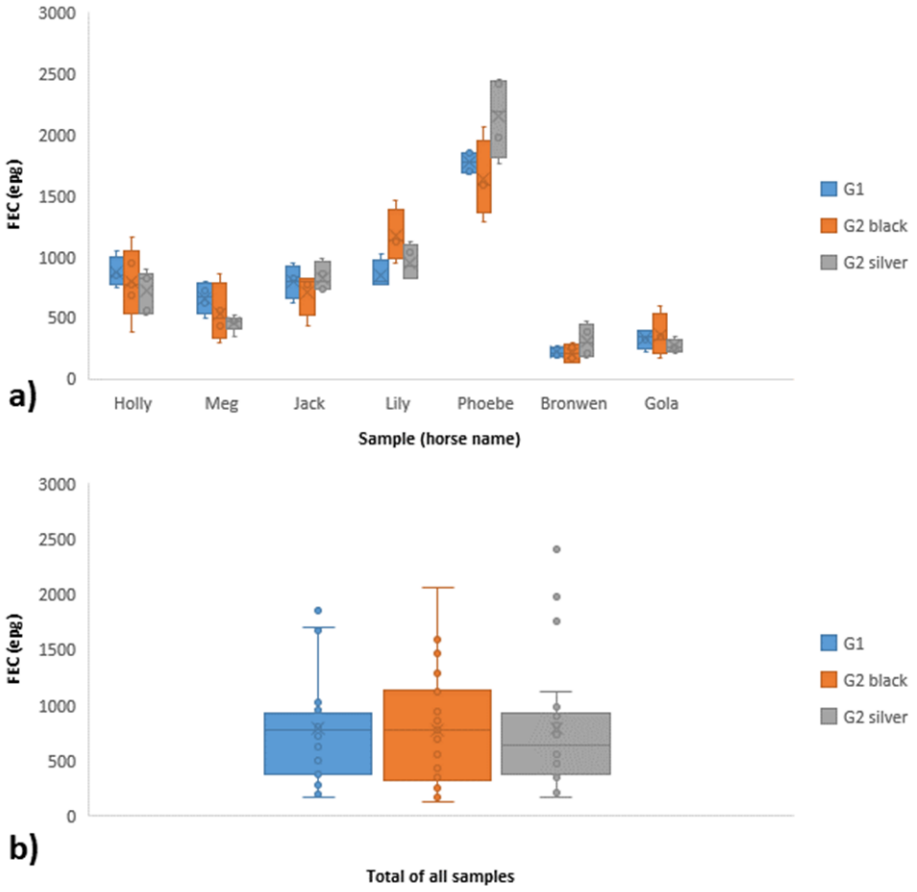

Figure 2-22 Comparing filters imaged using the G2 cassette and imaged with the Micro-I a) Data comprises each G2 count: epg returned from each filter size (height on the y axis of the boxes) and also the repeatability for each filter size with each sample (the size of each box plus whisker). b) Data comprises the total counts for all samples: epg returned from each filter size (height on the y axis of the boxes) and also the repeatability for each filter size.

The results in Figure 2-22a show no clear difference between filter sizes. Figure 2-22b demonstrates that the silver filters were more consistent with the control

values, yet Figure 2-23 confirms that the percentage accuracy of the two filter sizes was almost identical, running at approximately 100% of the G1 control values across a wide range of FEC levels.

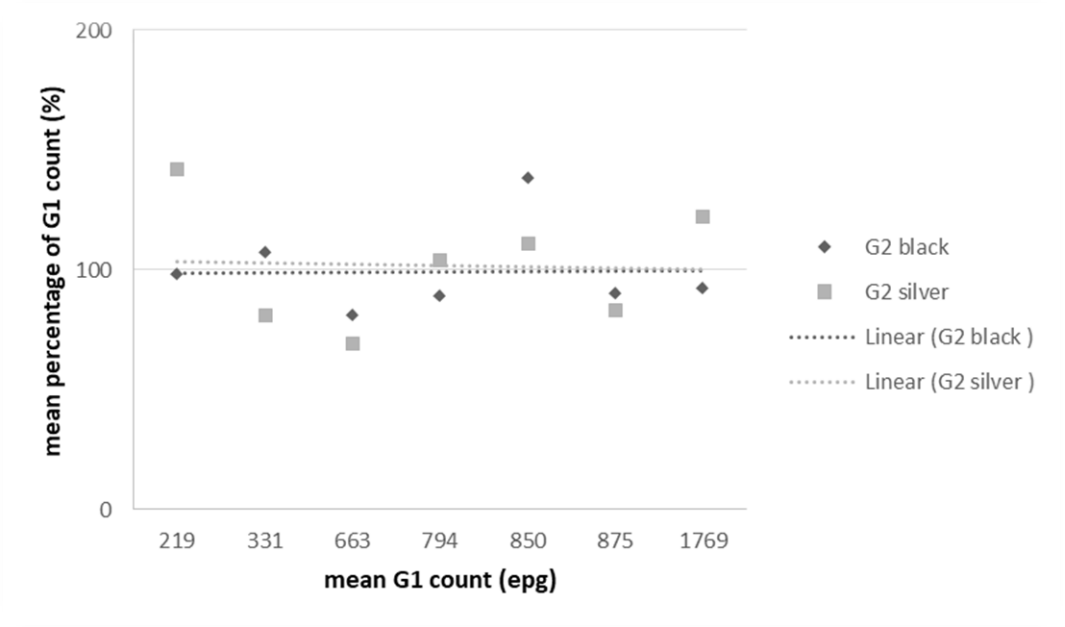

Figure 2-23 Percentage accuracy of black vs silver filters imaged using the G2 cassette and Micro-I. Each pair of data points represents the mean of two G2 counts for each filter size, for each sample, with the percentage accuracy compared to the mean G1 control for that sample plotted against the FEC as determined by the G1 control.

2.14.4 Filter sizes - discussion

In four out of the seven horses, the black filters gave higher FECs than the silver filters, with the reverse being true for the remaining three horses. When comparing all the results, the silver filters gave results that were slightly more consistent with the G1 control values. The mean G2 results for each horse expressed as a percentage of the G1 mean for that horse, showed that both filter sizes gave, on average, similar results to the G1 values therefore it was concluded that there was no clear benefit to using the finer black filters over the silver filters. In fact, the silver filters showed a slightly better performance over the black filters (102% of G1 control values for the

silver filters, 99% of the G1 control values for the black filters). Given the existing FECPAK^{G2} kits are supplied with silver filters, it was decided that there was no reason to change this. It was decided that the protocol to go forward for validation would use the silver filters.

2.15 CONCLUSION

In order to inform anthelmintic treatment decisions in horses, a method of detecting parasitic nematode infections is necessary. The most commonly used method is the faecal egg count, or FEC. Many protocols for performing FECs currently exist, most of which involve mixing a sample of equine faeces with a flotation solution and visualising the nematode eggs via light microscopy, such as the McMaster method (MAFF, 1986), FLOTAC (Rinaldi *et al.*, 2014) and Mini-FLOTAC (Levecke *et al.*, 2012b). Alternative FEC methods have recently been developed that avoid the need to visualise FEC preparations under a separate microscope, such as the Parasight system (MEP_Equine_Solutions, 2019) and the FECPAK^{G2} currently in use in ruminants (Rashid *et al.*, 2018) and humans (Ayana *et al.*, 2018). The FECPAK^{G2} involves mixing a faecal sample with a flotation solution as in the other methods mentioned but the preparation produced is then imaged in a cassette specifically developed for the Micro-I imaging device. The images are uploaded via the internet where they are counted by specialist technicians and the FEC returned to the customer electronically. Given the requirement to improve equine FECs, the aim of this Chapter was to determine a protocol for preparing equine faeces, that would give optimum results when imaged using G2 cassette and the Micro-I.

Compared to the McMaster method, which was the original faecal flotation method (MAFF, 1986), the FECPAK^{G2} method uses a larger faecal volume, which is

important as it reduces the error rate caused by the random distribution of helminth eggs within the faeces (Denwood *et al*., 2012). The earliest work described in this section confirmed that care should be taken with the initial sub-sampling of this faecal slurry, as equine faeces behave slightly differently than those of sheep. This was an important finding, as it prevented errors being introduced in the very first step of the protocol. Spooning the sub-sample rather than pouring it was found to produce a more representative sub-sample, and therefore gave more accurate results.

The volume of slurry to be used, which affected the sensitivity of the final test, was investigated. The initial idea of varying the volume led to comparisons between the selected volumes of 12 ml and 20 ml. These were compared using slides and using the G2 cassette in the Micro-I. Although the more sensitive 20 ml preparation performed well when counted using a slide, it did not perform as well when counted using the G2 cassette and Micro-I and so the 12 ml preparation was decided upon for the final protocol.

The optimum sedimentation time and accumulation time were decided upon for the 12 ml preparation, with some additional work on sedimentation and accumulation times performed for the 20 ml preparation which ended up not being used at this point. A sedimentation time of 30 minutes, and an accumulation time of six minutes were settled on for the final protocol. Finally, the optimum choice of filter sizes from those provided by Techion was determined and it was decided that the silver filters should be used.

From the work carried out described above, it was decided that the final protocol for a G2 test would be as follows:

- Mix faeces with water in the ratio 1:4 and mix to form a uniform slurry
- Spoon 12 ml slurry into a sedimentor and fill with water, invert three times to mix
- Pre-filter using the FECPAK cylinder fitted with a 1,000 µm filter and return to the sedimentor
- Allow to stand for 30 minutes, then discard the supernatant
- Add 80 ml saturated NaCl solution to the sediment and pour into a FECPAK^{G2} cylinder fitted with 600 µm and 425 µm (sliver) filters, invert three times to mix
- Fill both wells of the FECPAK^{G2} cassette from the cylinder, mixing between each aliquot
- Allow the cassette to stand for six minutes for the eggs to accumulate, then image using the Micro-I

This protocol then went forward to the validation stage, to be tested against the G1 protocol for multiple samples.

3 VALIDATION OF THE FECPAK^{G2} FOR EQUINES

3.1 INTRODUCTION

The FECPAK^{G1} method (Presland *et al.*, 2005) is a commercial faecal egg counting (FEC) method based on similar principles to the McMaster method (MAFF, 1986). FECPAK^{G1} differs from the McMaster method by exploiting a larger microscope slide in order to improve sensitivity. The sensitivity of a FEC method refers to the multiplication factor applied to each helminth egg observed within the count and represents the lowest infection level that can be detected by a single test. The FECPAK^{G1} method was initially developed for use in ruminants and has been successfully utilised in the ruminant sector since its launch in 1993 (Godber *et al.*, 2015; McCoy *et al.*, 2005) The FECPAK^{G1} has since been adapted for equines from 2004, and is now widely utilised in the equid sector (Presland *et al.*, 2005). Therefore, the FECPAK^{G1} (G1) represents an excellent tool as a comparative FEC system for validating the FECPAK^{G2} (G2) with the Micro-I automated imaging system and cassette. If the FECPAK^{G2} performed as well as the FECPAK^{G1}, it would represent an important improvement since it would enable owners to perform FECs on their animals whilst avoiding the common problem of mis-identification of eggs, which can lead to a significant over-estimation of strongyle egg counts as noted by McCoy *et al.* (2005).

Earlier optimisation work in this thesis (Chapter 2) had determined that the initial faecal slurry was best spooned into the FECPAK^{G2} sedimentor, in order to account for the different consistency of equine faeces compared to ovine faeces. A sedimentation time of 30 minutes was determined to be the optimum for nematode egg recovery, and filtering with 600 µm and 425 µm filters in the FECPAK^{G2} filter cylinder was deemed to be sufficient for removing debris to allow the nematode eggs to be imaged using the Micro-I and cassette. An accumulation time of six minutes

was noted to be sufficient to allow all the nematode eggs to float into the 3 mm field of view of the Micro-I. Two different concentrations of faecal slurry preparation were investigated during the optimisation work, one based on the sheep protocol which used 12 ml of the faecal slurry, and a more sensitive protocol which utilised 20 ml. Initially, the more sensitive 20 ml protocol was chosen for validation. This would be a more valuable test, with a sensitivity of 26 epg, than the 12 ml protocol (43 epg), and would therefore enable resistance monitoring to be undertaken using fewer replicates. However, when attempting to validate the FECPAK^{G2} cassette system using the 20 ml protocol, the initial results were poor. The validation work was re-attempted using the 12 ml protocol after discussion with the industry partner Techion Ltd, who feared that the more concentrated 20 ml preparation may have been unsuitable for imaging using the Micro-I.

The FECPAK^{G2}, like the G1, was initially developed for use in ruminants albeit in this case specifically for sheep and has since been successfully employed for FEC analysis. Therefore, in order to determine if the use of the FECPAK^{G2} would be as accurate for monitoring parasite infections in horses as the G1 method, faecal samples from horses from both the UK and New Zealand were processed using each of the two methods. Repeat counts on each faecal sample were performed with each method, to determine the repeatability of the G2 method and how this compared to the G1. The two methods were then compared for each sample, to assess the relative accuracy of the G2 method over the range of egg shedding levels. If variability and FECs produced were similar between the two methods, the G2 would represent an improvement over the slide-based G1 method due to its ease of use for the end user. The aim of this chapter therefore, is to validate the FECPAK^{G2} as an acceptable method of performing faecal egg counts on horses. This task would be accomplished

by comparing FECs produced by the G2 method with those produced from an existing FEC method, the G1.

The validation initially commenced with the 20 ml protocol, but as this performed poorly when imaged in the cassette and Micro-I compared to when counted on a FECPAK^{G1} slide, the procedure was changed to validate using the 12 ml protocol instead.

3.2 CHAPTER AIMS

- Validate the optimised FECPAK^{G2} equine test against an established FEC method, the FECPAK^{G1}

3.3 MATERIALS AND METHODS

3.3.1 Validation 12 ml protocol

Faecal samples were collected from 92 horses in Wales which had been volunteered as participants by their owners. From each horse, 60 g of faeces was mixed with 240 ml water to produce a uniform slurry. A G1 preparation was made from each faecal slurry and two slides counted. Forty two of these horses had FECs of zero epg and thus their samples were discarded. Fifteen samples were also discarded due to their having FECs of less than 90 epg, which would represent only one egg on the G2 test. It was decided that at least two eggs should be expected in the G2 test before it would be a useful comparison to the G1 for validation purposes, as two images are produced for each test. For the remaining 40 samples a further G1 preparation was made and again two slides counted. The means of these four G1 counts were calculated and formed the control values.

From each sample two G2 preparations were made using 12 ml of faecal slurry. The first 26 samples were measured by spooning the slurry into the sedimentor up to the slurry line and the final 14 samples were measured using a 12 ml scoop which became available during the latter part of the validation. The faecal slurry was spooned into the sedimentors as the most accurate method developed during the optimisation work to ensure accurate subsampling of the faecal slurry. All sedimentors were filled with water to the fill line, pre-filtered using a 1,000 µm filter and left to stand for 30 minutes. The supernatant was then discarded and the sediment mixed with 80 ml saturated NaCl solution and poured into a FECPAK^{G2} cylinder fitted with 600 µm and 425 µm (silver) filters which had been selected as the best ones to use during the optimisation work. An aliquot was used to fill each well of two FECPAK^{G2} cassettes and these were image captured and marked up using the Micro-I.

3.3.2 Statistical analysis

The aim of this work was to prove that the G2 method produced results comparable to those of the G1, in order to validate the test as an effective tool for performing equine FECs.

At this point, a power equation was performed to determine the minimum number of samples needed for a statistically significant validation.

$$n \geq \left(\frac{(t_1 + t_2) * \sqrt{2} * CV}{d} \right)^2$$

In the above equation:

n = sample size needed

t_1 = t value for (P<0.05, error d.f.) = 2

t_2 = t value for (P=2(1-p), error d.f.) where p = the chance of success. Aiming for a 90% chance of success, thus p = 0.9 and t_2 = 1.3

CV = coefficient of variation, and the figure for CV of the G1 counts was used (78%).

d = "treatment success" i.e. if G2 was 95% of G1 that would be considered a success.

Substituting numbers into the equation gives:

$$n \geq \left(\frac{(2 + 1.3) * \sqrt{2} * 78}{95}\right)^2$$

giving n ≥ 14.68 Therefore, to give a 90% chance of identifying at least a 95% similarity between the G1 and G2 counts with p < 0.05, at least fifteen replicates should be used. After having discarded the results from nine samples that were over seven days old when tested, there were 17 sedimentor measured replicates and these were considered sufficient to proceed with the validation statistics. In order to increase the statistical robustness of the analysis, additional data were obtained from Techion Ltd.NZ. Faecal samples from 22 horses were processed in the same way as the UK samples described in section 3.3.1 "Validation 12 ml protocol " with the exception that the G1 preparations were counted only once.

Statistical analysis was carried out using SPSS version 22.0 (IBM computers Ltd). A Pearson correlation was calculated on the UK data, the New Zealand data and the combined data to test the correlation between the G1 egg counts and the G2 egg counts. Repeated measure ANOVA (rmANOVA) was used to determine the

repeatability of the FEC within each method in the U.K. and New Zealand samples. A rmANOVA was then performed to compare the mean G1 epg with the mean G2 epg (within-subject factor) and the UK and NZ data (between-subject factor) to determine whether the sampling methods differed and if the country in which they were tested affected this outcome. Effect size is reported as partial eta squared (η^2_p). Mauchly's Test of Sphericity was used to determine homogeneity of variance for rmANOVA, when heterogeneity was found the more conservative Greenhouse-Geisser correction was used.

Percentage deviation from the mean G1 count of a sample was calculated separately for each G2 count using the formula (G2 eggs expected – G2 eggs seen)/ G2 eggs expected × 100, where G2 eggs expected was: mean G1 eggs seen in that sample / 45 × 25 to take account of the different sensitivity of the G2 versus the G1 test. The percentage deviations were converted to positive values, so that an under-reading test would not compensate for an over-reading test. A Pearson correlation was performed on these percentage deviations against FEC from the original G1 egg count to determine if the relative accuracy of the G2 method differs with different levels of egg shedding.

3.3.3 Validation 20 ml protocol

Faecal samples were taken from nine horses. From each horse, a sample of 50 g faeces was mixed with 200 ml water to make a uniform slurry. From this faecal slurry, two G1 preparations were made according to the standard Techion Ltd protocol (Presland *et al.*, 2005) (Appendix one). Each preparation was used to fill three replicate G1 slides and these were counted using light microscopy at 40 × magnification. The mean of these six counts for each of the 9 equine faecal samples formed the control values.

From each slurry, two FECPAK^{G2} sedimentors were prepared using 20 ml slurry and filled with water to the fill line. The liquid from each was pre-filtered with a 1,000 µm filter, returned to the sedimentors and allowed to stand for 30 minutes. After 30 minutes sedimentation the supernatant was discarded. Eighty ml saturated NaCl solution was added to the sediment and the liquid transferred to a FECPAK^{G2} cylinder fitted with 425 µm and 250 µm filters. An aliquot from each was used to fill a G1 slide which was counted using light microscopy at 40 × magnification and also a G2 cassette which was imaged using the Micro-I. All Micro-I images were marked-up for helminth eggs and all marked up images were independently verified by Techion New Zealand.

3.3.4 G2 cassette imaging assessment

Initially, G2 cassette imaging issues were detected by repeatedly feeding the same cassettes through the Micro-I. Whilst optimising the 20 ml protocol, investigation into a longer accumulation time was performed. Two G2 cassettes were prepared from a single faecal slurry using protocol outlined in Section 3.3.3, allowed to accumulate for six minutes and then fed through the Micro-I one after the other. G2 cassette one was then immediately re-imaged, followed by G2 cassette two. This process was repeated resulting in three replicate sets of images for each G2 cassette at 6, 12 and 18 minutes accumulation time.

In addition, a further eight G2 cassettes were prepared as the two above images were captured using the ’timed capture‘ software (Techion Ltd) utilised in the optimisation work (Chapter 2), to capture eight images of each G2 cassette well 30 seconds apart. This software also captured the individual focused images used to create the final FECPAK^{G2} stacked image, so these could also be examined.

3.4 RESULTS

3.4.1 Validation 12 ml protocol

As described in section 3.3.1, the validation samples were initially measured by spooning the faecal slurry into the sedimentor up to the slurry line, and later by utilising a 12 ml scoop which became available. Figure 3-1 demonstrates the mean G1 and G2 cassette counts for each equine FEC performed together with the percentage accuracy of the G2 compared to the G1 counts for samples from the forty horses. It can be observed that for the first seventeen samples (Figure 3-1 Blue data points) the mean accuracy of the G2 test compared to the G1 controls was approximately 100%. However, samples 18 to 26 (Figure 3-1 red data points), demonstrated the G2 protocol on samples older than 7 days, performed markedly worse compared to the G1 controls. As described in 3.4.1.1 (Effect of sample age on G2 performance), these samples were more than a week old, and the decision was made to exclude old samples from the validation work. These samples had been used owing to the difficulty in obtaining faecal samples from horses with a FEC of sufficient magnitude for the validation, as described in section 3.4.4.

The next fourteen samples, however, showed a sudden drop in G2 relative accuracy to approximately 70% of the G1 control values, and analysis was performed to try to determine the cause. Initially, it was postulated that the size of the horses may have been the cause of this reduction in performance of the G2 test, and the data analysis for this hypothesis is included in section 3.4.1.2 (Effect of height of animals on G2 performance). However, once the significance of the change in protocol became apparent, separate values were produced for the results obtained using the 12 ml

scoop versus those obtained by measuring the slurry using the line on the sedimentor, as shown by the green data points in Figure 3-1.

Numerical data is contained in section 7.6 of the appendix.

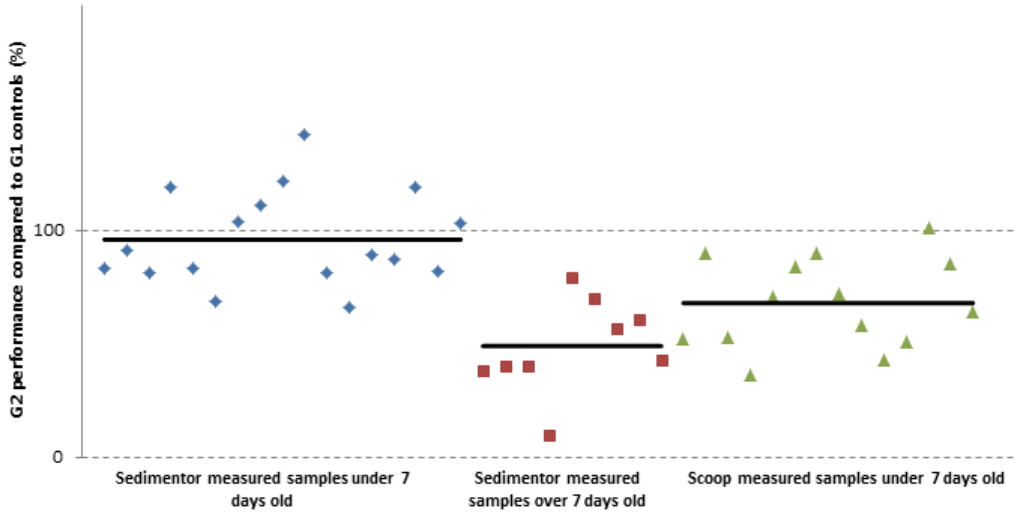

Figure 3-1 The. performance of the optimised 12 ml G2 test compared to G1 controls over the validation process for equine faecal samples. Blue data points represent samples measured with a sedimentor under seven days old when tested, red data points represent samples measured with a sedimentor over seven days old when tested, green data points represent samples measured with 12 ml scoop (under seven days old when tested). Black lines represent the mean relative accuracy of each set of samples.

3.4.1.1 Effect of sample age on G2 performance

The frequency with which samples would return a zero count had been under-estimated, and hence many of the samples collected were inadequate for the validation work. In order to maximise the data available, nine samples retained from the anthelmintic resistance testing were re-used for the validation work. These nine samples had been stored at 4ºC for eight days or more.

The G2 data from these older samples produced much lower FECs than the corresponding G1 control results, with a drop in G2 relative accuracy from 96% to 49% of the G1 control values. This represented a significant difference between the G1 control values and the G2 counts, (two-tailed t test $p < 0.01$). It was interesting to note that the G2 performance compared to the G1 fell with samples that were over a week old when they were tested, not simply the FEC values themselves. Actual G1 FEC values on the old samples dropped by an average of 16% (±29%), although this difference was not significant (two-tailed t test $p = 0.2$).

When the comparative G2 accuracy of all faecal samples was tested against the age of the sample (storage), the trend was for the G2 accuracy to drop compared to the G1 controls as the samples became older (Figure 3-2a). This phenomenon occurred despite the fact that the G1 control values were also prepared from the old samples, rather than using the FEC data obtained when the samples were fresh. The data were re-analysed using only samples up to seven days old (Figure 3-2b) from which it can be seen that G2 accuracy compared to the G1 control figure was unaffected by the age of the sample.

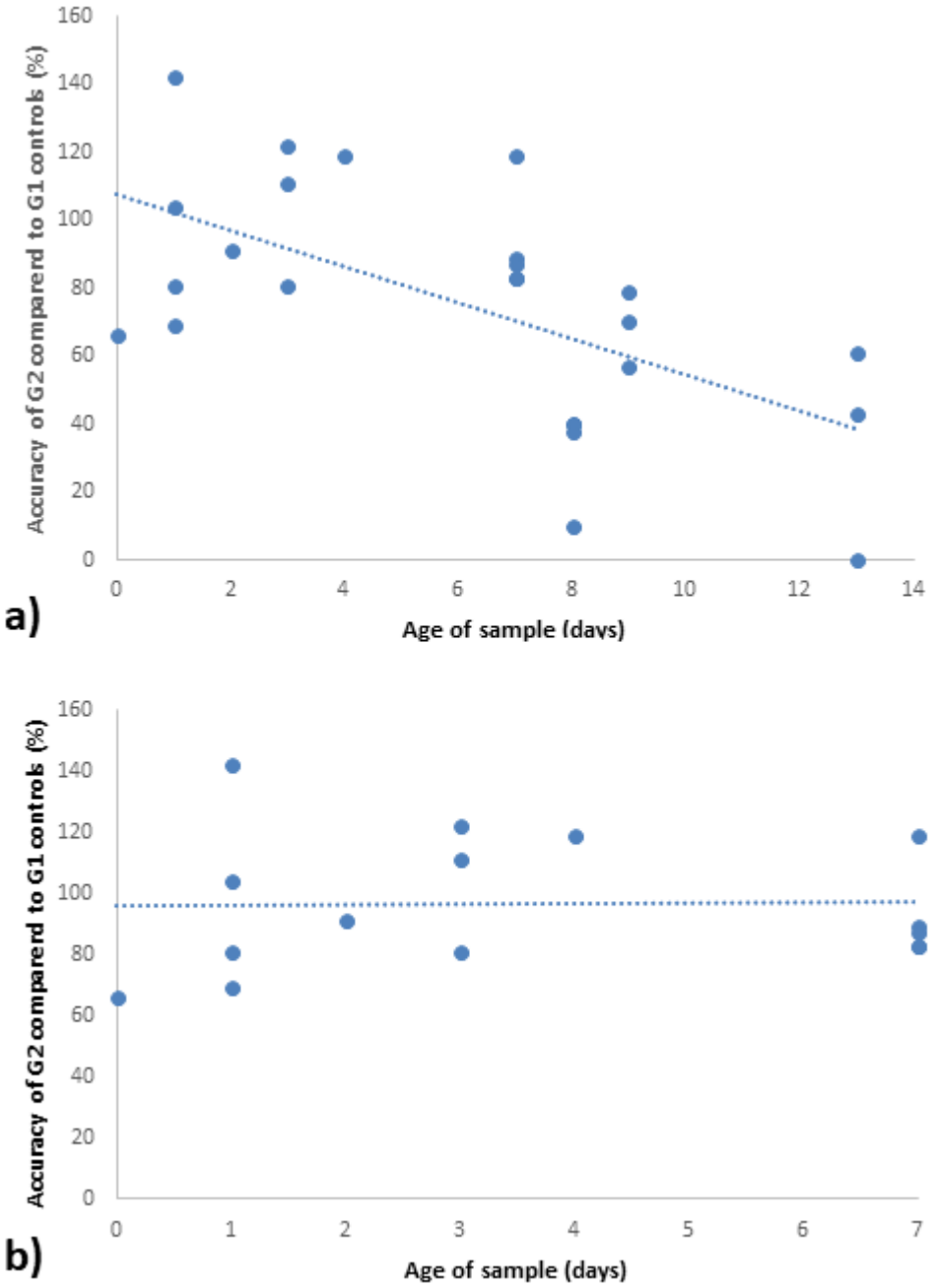

Figure 3-2 Accuracy of G2 count compared to G1 controls plotted against sample age for equine FECs. Individual data points represent mean G2 FEC as a percentage of mean G1 FEC for each sample, plotted against sample age. a) Using all samples tested. Trendline shows accuracy of G2 compared to G1 dropping off as sample age increases. b) Using samples up to seven days old. Trendline shows accuracy of G2 compared to G1 steady at nearly 100%.

Due to this discovery, it was decided to exclude the data from the validation from the nine samples that had been tested at eight days old or older. After this decision was

made, two further fresh samples were processed and the G2 accuracy compared to the G1 controls improved back to previous levels.

3.4.1.2 Effect of height of animals on G2 performance

After excluding faecal samples stored at 4^{o}C for over 7 days, when further samples were tested the relative accuracy of the G2 method suddenly dropped to a mean 70.5% of the G1 control values. This represented a noticeable drop-off in relative accuracy, even when using fresh faecal samples. Figure 3-3a demonstrates a trend towards greater relative accuracy as the height of the animals increased. Even with the older samples excluded from the dataset, this trend was still apparent (Figure 3-3b).

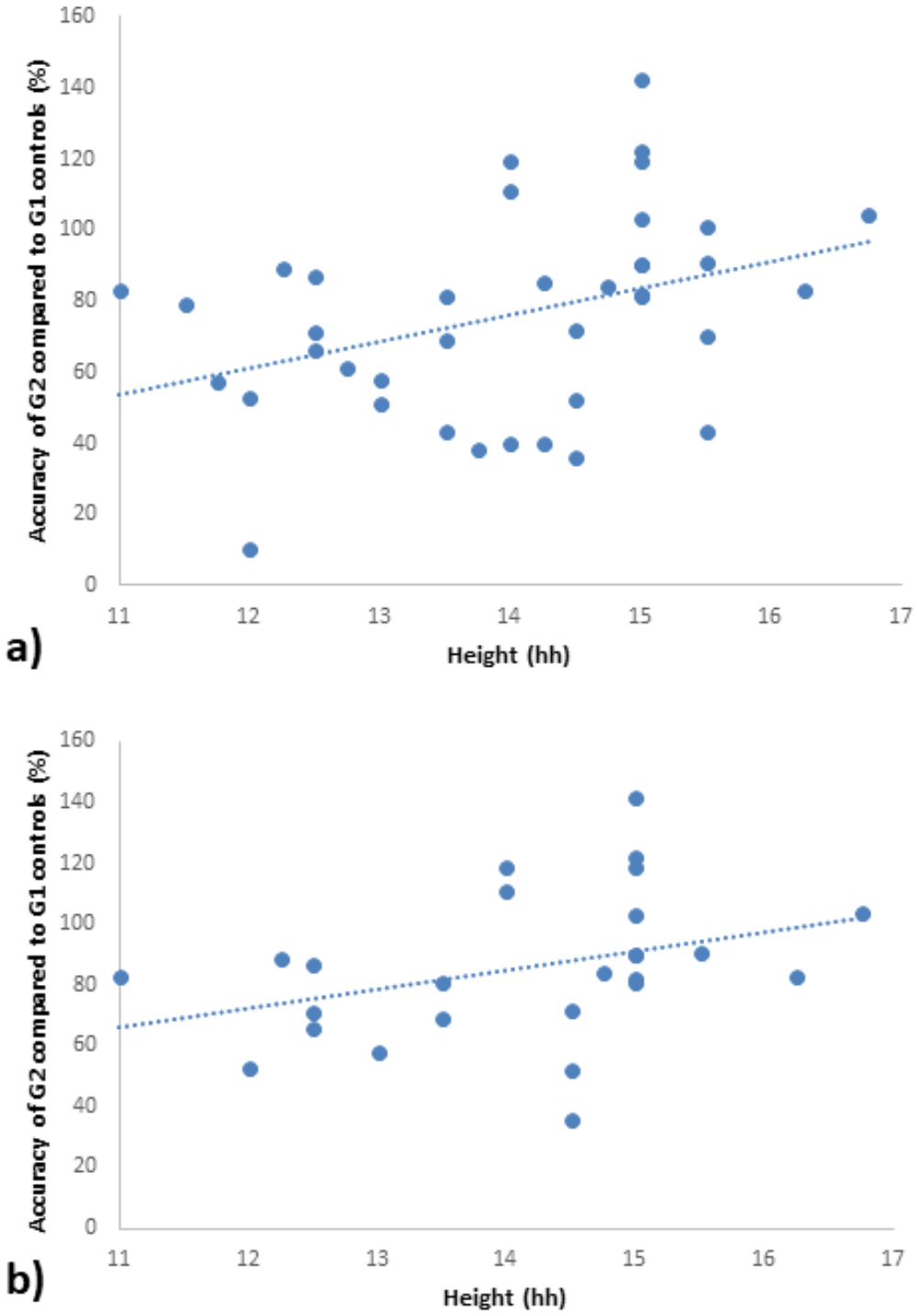

Figure 3-3 Accuracy of G2 compared to G1 controls compared to the height of the animal Individual data points represent mean G2 FEC as a percentage of mean G1 FEC for each equine faecal sample, plotted against height of animal. a) Including samples over a week old when tested. b) Excluding samples over a week old when tested. Both trendlines demonstrate relative accuracy of G2 compared to G1 increasing for larger animals.

The datasets were divided into two categories: animals below 15 hh and animals of 15 hh and above. The mean relative accuracy of the G2 method for animals under 15 hh was 75% and it can be observed (Figure 3-4a) that the trend line was almost horizontal suggesting that this mean was consistent for the different horse heights sampled between 11 hh and 14.3 hh. The mean G2 relative accuracy for animals 15 hh and over was 101% of the G1 control values (Figure 3-4b). Most of these horses were actually exactly 15 hh but even so the trend line is still almost horizontal showing that the mean G2 relative accuracy of samples from larger horses was also consistent with that value regardless of increasing height.

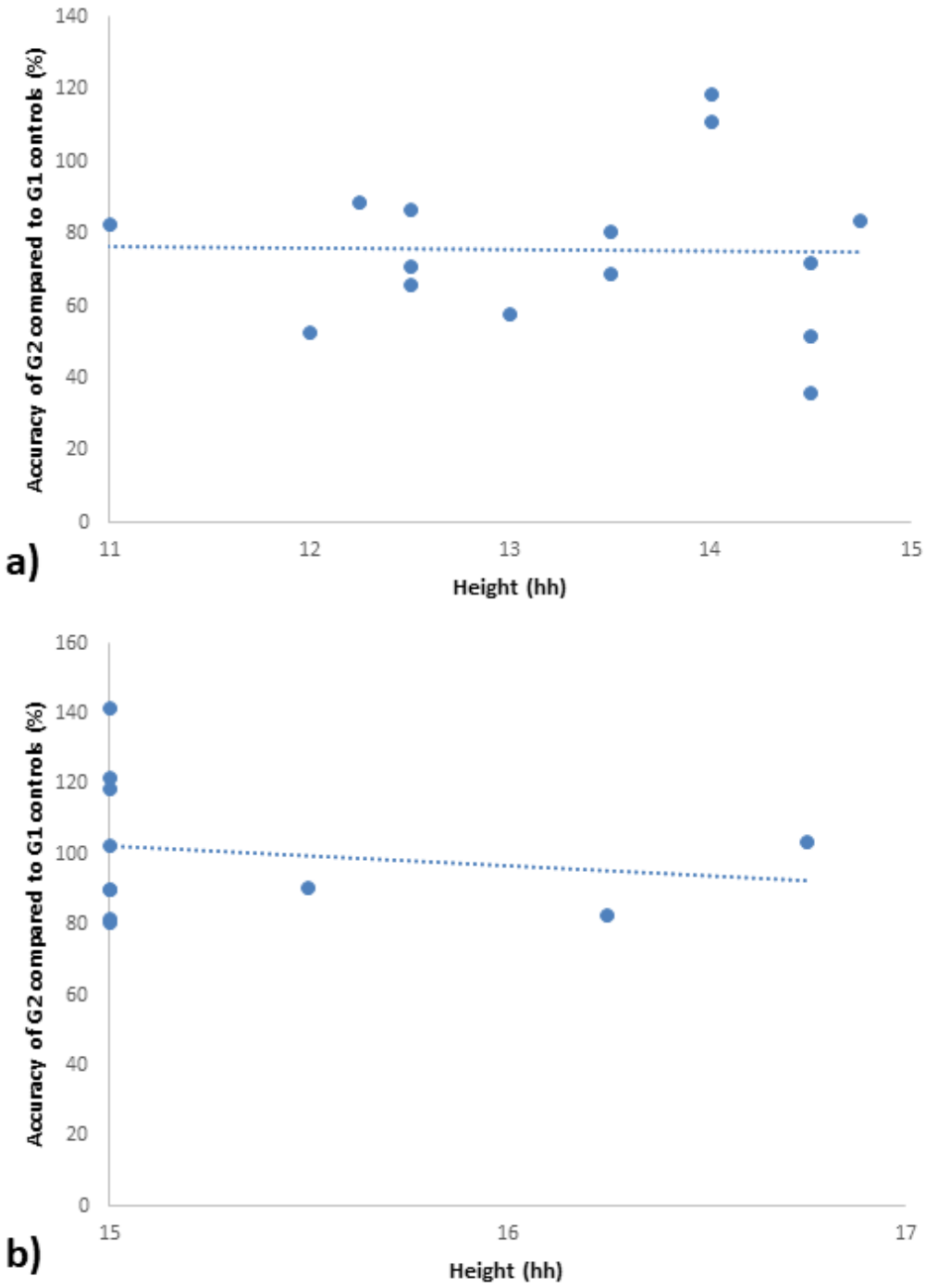

Figure 3-4 Accuracy of G2 compared to G1 controls plotted against the height of the animal, excluding samples over a week old when tested a) Including only animals under 15 hh. Showing a mean G2 relative accuracy of 75% of G1 control figures b) Including only 15 hh and over. Showing a mean G2 relative accuracy of 101% of G1 control figures.

3.4.1.3 Change in protocol – scoop measure vs sedimentor measure

Another potential cause for loss of relative accuracy of the G2 test was a preserved minor change in validation protocol. The existing data were re-examined and it was discovered that the decline in performance of the G2 test corresponded with the point at which the 12 ml scoop became available for use, in addition to coinciding with a drop in the size of the animals providing the samples. The samples processed using the 12 ml protocol and measured using the sedimentor returned a mean G2 relative accuracy of 96% of the G1 counts, whereas those measured using the scoop had a mean relative accuracy of 68% of the G1 (Figure 3-5).

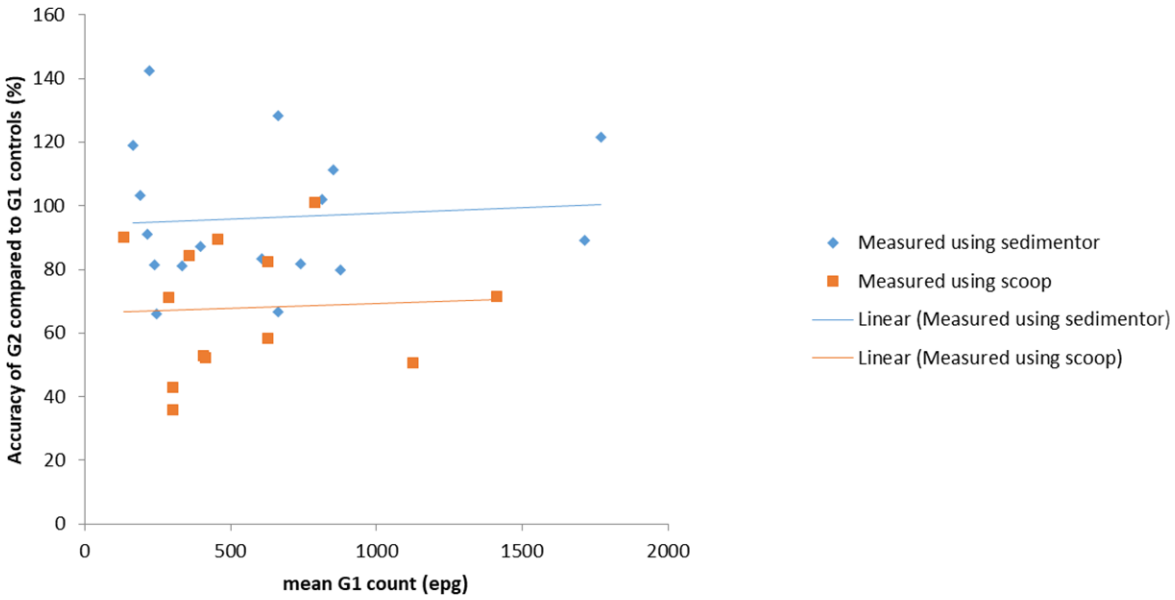

Figure 3-5 Percentage accuracy of G2 compared to G1 control values for samples measured using the scoop vs the sedimentor. Blue data points are sedimentor measured samples, showing a mean relative accuracy of 96% of the G1 controls, orange data points are scoop measured samples showing a mean relative accuracy of 68% of the G1 controls. Both trendlines demonstrate that these accuracies are fairly consistent across a range of FEC levels.

3.4.2 Validation 20 ml protocol

During the optimisation phase, a protocol using 20 ml of faecal slurry rather than the originally suggested 12 ml was developed. This would deliver a test with a lower multiplier (sensitivity) enabling lower infection levels to be detected. As demonstrated in Figure 3-6, in every case the G2 preparations gave higher FEC epgs when counted on a slide (G2 Slide) following the G2 protocol rather than the G1 control counts. However, when this same G2 preparation was image captured using the Micro-I (G2 Cassette) the FEC epg counts were consistently lower – lower, in fact, than those from the controls.

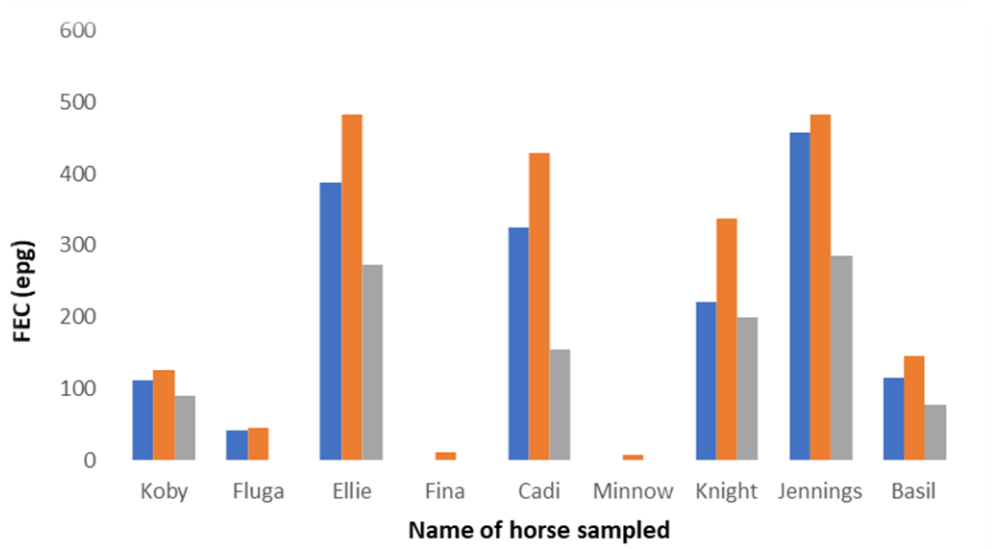

Figure 3-6 Validation of the developed 20 ml FECPAK^{G2} protocol for equine FECs. FECs were generated from nine horses using three different methods; Blue bars representing G1 control FECs (mean of six counts per horse), orange bars representing a G2 Slide (FEC based on a slide count; mean of two counts per horse) and the grey bars representing a G2 Cassette (FEC imaged using the Micro-I; mean of two counts per horse).

The average FEC expressed as epg across the nine horses was 185 epg (range 0 epg to 459 epg) when processed using the G1 control method, and 230 epg (range 8 epg to 483 epg) when processed with the G2 slide count. However, when these same G2 preparations were imaged using the FECPAK^{G2} Micro-I and G2 cassette, the average FEC was only 120 epg (range 0 epg to 286 epg) representing a 35% reduction in epg compared with the control values and a 48% average reduction during the G2 cassette imaging process (difference in egg count when counting using the G2 cassette versus counting on the slide). These differences were significant in every case (two-tailed t-test, $p < 0.05$).

3.4.3 Micro-I imaging assessment

Subsequent to the validation of the FECPAK^{G2} protocol, work was undertaken in an attempt to increase the sensitivity of the test. During this work, several cassettes were repeatedly imaged by the Micro-I. It became apparent that the same cassette re-imaged multiple times did not always return the same egg count. Thus, the aim of this investigation was to determine the cause.

Initially, two prepared cassettes of equine FECs were imaged in triplicate being fed in turn into the Micro-I. Following the repeat imaging of cassettes it can be noted that not all helminth eggs present within the faecal samples were being correctly imaged within the Micro-I (Table 3-1). For example, in cassette two of faecal sample one, there were at least ten eggs across the two wells, given there were four eggs imaged in well one and six eggs imaged in well two captured on different occasions. However, despite there being a minimum of 10 eggs present in both wells the maximum number imaged at any one time by the Micro-I was six.

Table 3-1 FECs from repeat imaging of G2 cassettes, demonstrating different numbers of eggs imaged from the same G2 cassettes on when imaging was repeated (image IDs refer to images uploaded onto the FECPAK server). [A]The maximum number of eggs observed in well one and well two combined.

	Cassette 1			Cassette 2		
	Well 1	**Well 2**	**Image ID**	**Well 1**	**Well 2**	**Image ID**
	4	4	151849	4	2	151850
Sample 1	3	4	151851	0	6	151852
	2	4	151853	0	4	151854
Total Eggs[A]	8			10		
	0	1	151860	8	5	151861
Sample 2	1	1	151863	9	4	151864
	1	1	151865	7	4	151866
Total Eggs[A]	2			14		

After this phenomenon became apparent, every G2 cassette used in the experiment was re-imaged in triplicate, and the same problem was observed repeatedly. As this problem was not a previously documented issue, a sample of sheep faeces was also imaged in order to see if the phenomenon was unique to horse samples and repeat images again produced differing numbers of eggs (data not shown).

In order to investigate the reason for the variation in egg numbers, G2 cassettes were re-imaged using the timed-capture software recording eight images without moving the G2 cassette in between image captures. Ten G2 cassettes were re-imaged in this

manner and a representative example of the results found is shown in Table 3-2. The entire set of results is shown in section 1.7 of the Appendix.

Table 3-2 Number of eggs observed in a single FECPAK^{G2} cassette, imaged eight times using timed-capture software (counts presented in consecutive order). Image references are the reference supplied when the image was uploaded to the FECPAK server. NB the results from one well only are shown, each cassette actually produced two such sets of images. Necessarily later images have a longer accumulation time than is standard.

Eggs seen	Image reference
0	153552
1	153553
3	153554
4	153555
1	153556
4	153557
4	153558
4	153559

In order to investigate the reason for the differing egg counts, the individual images were examined and it was noted that the stacking software underpinning the Micro-I image capture was often choosing out-of-focus parts of the images to form the final stacked image for image analysis and helminth egg mark up.

Representative images (Figure 3-7) of the data presented in Table 3-2 demonstrate such challenges with the imaging software imaging no eggs initially yet in later images of the same well capturing four helminth eggs. Specifically, Figure 3-7a demonstrates the centre of the well as seen on the stacked image. The stacked image is the only one that is visible when imaging the FECPAK^{G2} cassettes using the

normal laboratory procedure. This stacked image for image ID 153552 produced an egg count of zero. However, when the individual images that formed the stack were examined, it can be observed that there were three helminth eggs in focus within this area on one of the images (Figure 3-7b) which the stacking software had missed.

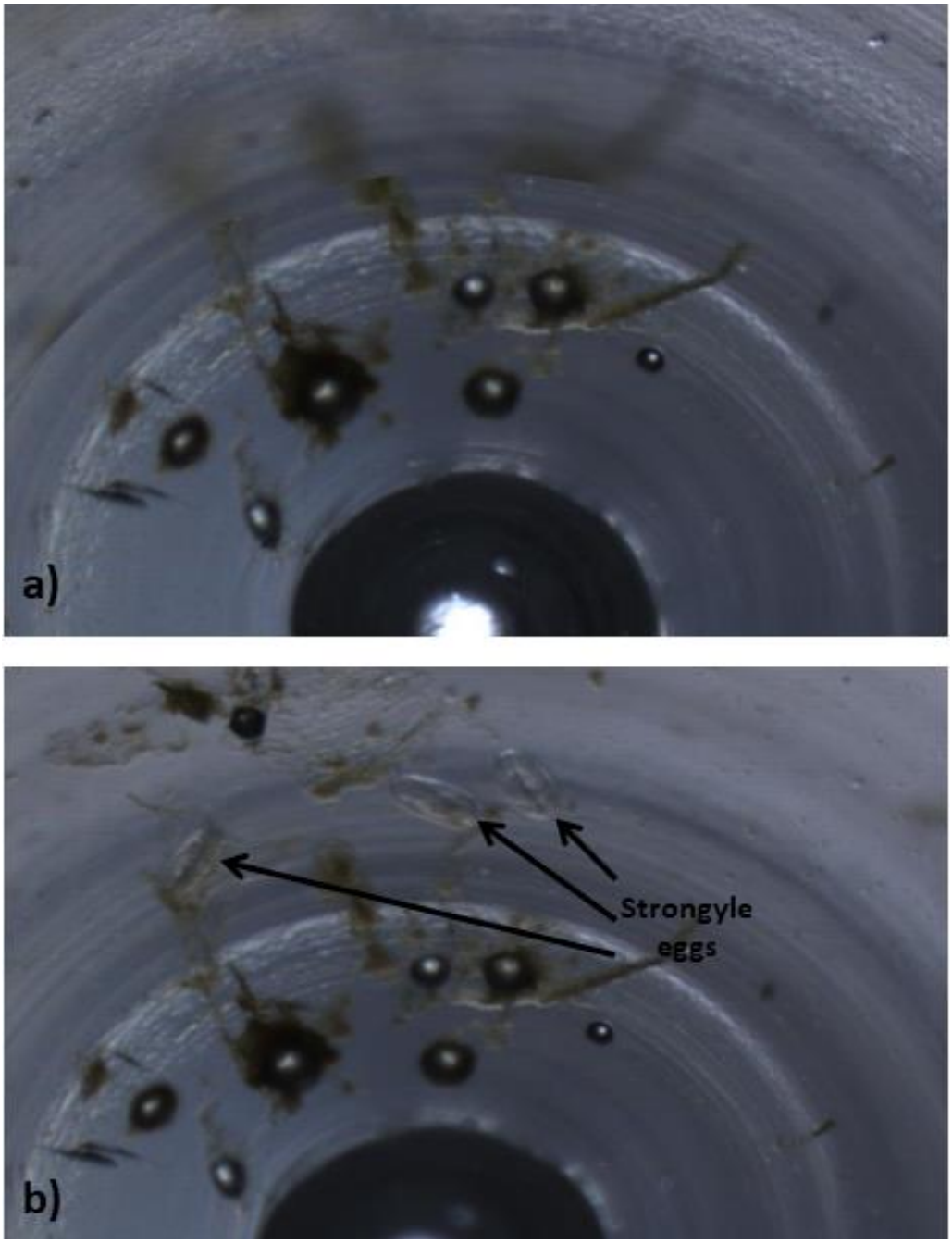

Figure 3-7 Image ID 153552 a) Focus stacked image showing no visible eggs. Bubbles and debris are visible in centre focus ring but second focus ring is blurred. b) Third image in stack for image 153552 showing three eggs (highlighted) in second focus ring. Bubbles and debris still visible in centre focus ring.

Following the discovery of the G2 cassette imaging challenges, it was decided to re-visit all existing experimental data where G2 Slide counts and G2 Cassette Micro-I

counts of the same preparation were available. For final the validation, the standard G1 protocol was used as a comparison to the G2 Cassette on the request of Techion. At the time the 20 ml protocol was being investigated, the control values were being produced by counting the G2 preparation on a FECPAK slide (G2 Slide). The values produced by the G2 Cassette fell short of the control values, and so the 20 ml protocol was abandoned. In total there were 67 different experiments where data were available for G2 Slide and G2 Cassette counts of the same preparation. Mean G2 Cassette value was 56% of the G2 Slide counts across all protocols that had been performed. The final 12 ml protocol that went forward for validation against the G1 control values gave a mean G2 Cassette value of 69% of the G2 Slide counts, and the 20 ml protocol gave a mean G2 Cassette value of 76% of the G2 Slide counts.

3.4.4 Statistical analysis

Figure 3-8 provides the fate of the samples from 119 horses recruited for the validation work. As some of the optimisation work was done in tandem with the validation, this led to ten samples being processed using protocols which differed from the final optimised protocol, so these results were not used in the final validation figures. Fifty seven of the samples returned FECs of less than 90 epg, which were not used for the validation given at least two eggs should be present in the FECPAK^{G2} images for comparison to be made. Seven owners who had promised samples did not provide them (these are marked “not given”), nine were over a week old when used for the validation work and therefore the results discarded, and a further five were discarded at over a week old without being tested. This left 31

samples, which together with the nine samples discarded due to age, made up the 40 samples referred to in Section 3.3.1.

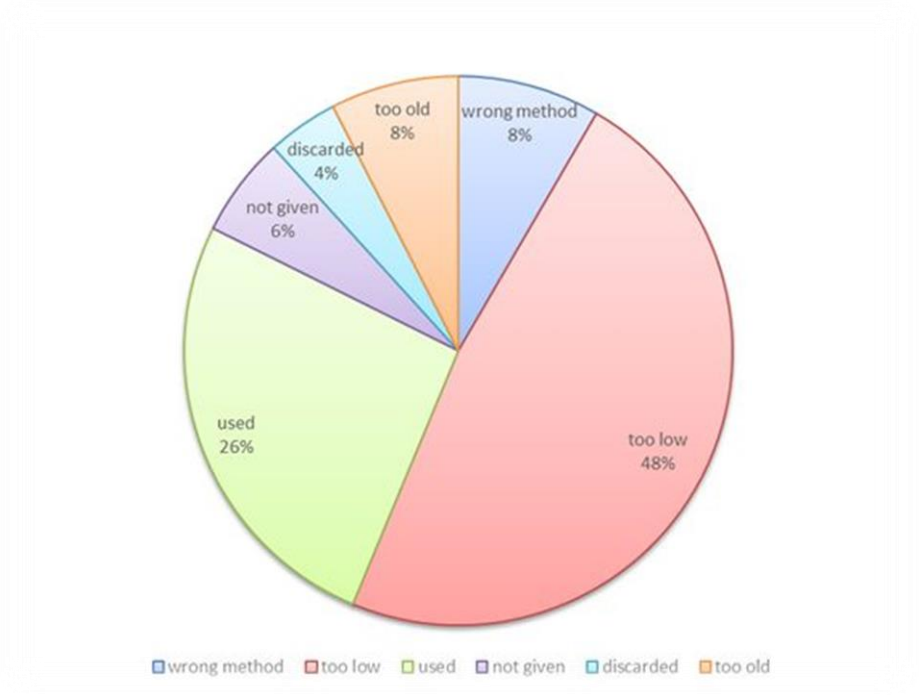

Figure 3-8 The fate of equine faecal samples intended for FECPAK^{G2} validation. In total 119 samples were recruited demonstrating that almost 50% of samples were unsuitable for validation work due to zero or low epg FECs (too low), 8% were processed with protocols which differed to the final optimised protocol, 8% were processed at over a week old and the results therefore discarded, along with 4% that were discarded at over a week old without being processed. Promised samples which were not given by the owners represented 6% of the total, leaving 26% of the samples available for the validation work.

There was a significant positive correlation between the mean G1 counts and the mean G2 counts for each sample of the combined dataset (r=0.971 (CI:0.956, 0.987), n=39, p<0.001) (Figure 3-9). This was also independently true for both the UK FEC samples (r=0.963 (CI:0.907, 0.990), n=17, p<0.001) and for the New Zealand FEC samples (r=0.974 (CI:0.962, 0.993), n=22, p<0.001).

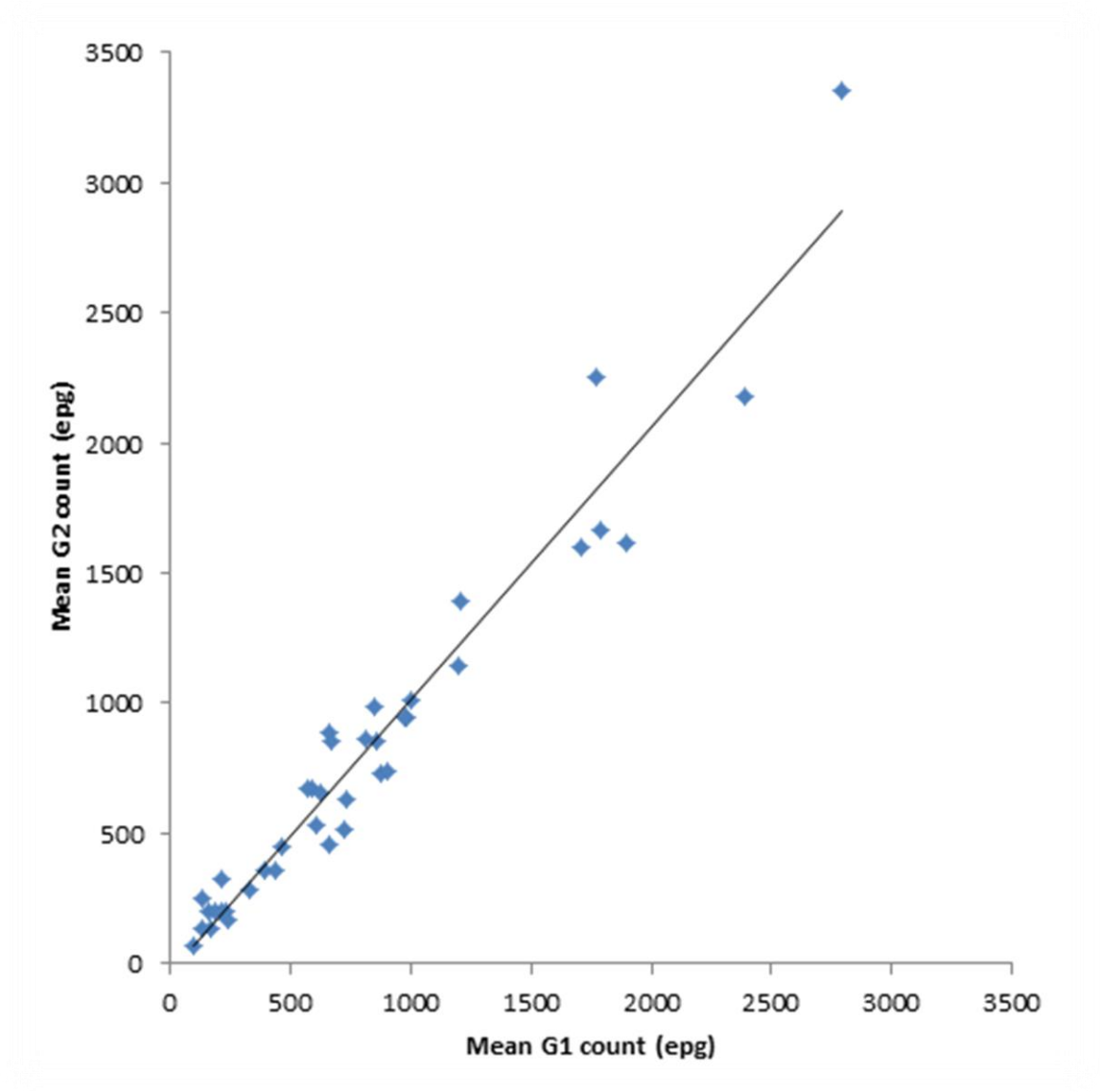

Figure 3-9 Comparison between mean G2 counts and mean G1 control counts for each sample, demonstrating a strong positive correlation with G2 counts running at very close to the control values. The mean percentage accuracy (mean G2 count as a percentage of mean G1 count, ± SE) was determined at 101 ± 4%.

It was important to determine whether FECs performed using the G2 were as accurate as those performed using the control method (G1). Using rmANOVA there was no significant difference between the repeat samples using the G1 or the G2 method in either the UK or the NZ data (Table 3-3). Repeatability was therefore similar with both methods.

Table 3-3 Repeatability of methods of equine faecal egg counting using the FECPAK^{G1} and FECPAK^{G2}. RmANOVA was used to test the repeatability of the G1 and G2 methods on FEC replicates of individual equine samples. The analysis demonstrates no differences between repeated sampling meaning that average FEC for each individual could be used to compare the G1 and G2 methods.

Method and Country	Degrees of freedom	F	P
G1 UK	1.9, 30.2	0.785	0.458
G2 UK	3, 48	0.743	0.532
G1 NZ	1, 21	2.253	0.148
G2 NZ	1, 21	1.115	0.303

It was also important that FEC for each sample was comparable between the two methods. Using rmANOVA there was no significant effect of the method used on the mean epg per sample ($F_{1,37}$ = 0.052, p = 0.821, η^2_p = 0.001). Therefore, the G2 method detected infection levels with efficacy equal to the G1 method. To examine any effect of country of origin of the data, the data from the NZ and UK were compared again using rmANOVA. Epg was also not affected by the country of origin of the data ($F_{1,37}$ = 2.084 p = 0.157, η^2_p = 0.053) and there was no significant interaction between the method used and the country of origin of the data ($F_{1,37}$ = 0.056 p = 0.814, η^2_p = 0.002).

The overall similarity between FECs on each sample was also calculated. The mean percentage accuracy (mean G2 count as a percentage of mean G1 count, ± SE) was 101 ± 4%. Importantly, relative accuracy of the mean G2 count was not significantly affected by FEC level (r = -0.251 (CI: 0.030, -0.472) p= 0.124 n = 39). Therefore, a lack of significant correlation between relative accuracy and G1 FEC indicates that the relative accuracy of the G2 method is not dependent on the FEC level.

Egg count per sample was equally consistent between the G1 and G2 methods whether looking at the UK data, the NZ data or the combined dataset. Importantly, there was no significant difference between egg count repeat samples using either method (Table 3-3) providing confidence that both methods were reliable and that they were consistently applied.

3.5 DISCUSSION

3.5.1 12 ml protocol

In total, 119 samples had been recruited and FECs performed in order to collect these 17 biological replicates. Losses were specifically due to low FECs, samples not being produced, or changes in the protocol that rendered the results unusable for the validation work as the methods used did not reflect the final optimised protocol. Total prevalence of positive FECs in this study was 37%, similar to the level of infection observed in a later study using the FECPAK^{G2} system on samples from horses at the Royal Welsh agricultural show, where 50% of the horses tested had a detectable infection (Tyson *et al.*, 2017) and is consistent with other research (Lester *et al.*, 2018; Nielsen *et al.*, 2018b; Relf *et al.*, 2013).

3.5.1.1 Effect of sample age on G2 performance

When using samples over a week old, it was noted that the relative accuracy of the G2 test dropped from 96% to 49% of the G1 control values. It would not be surprising if the FECs themselves dropped off as the samples aged, but as determining the overall accuracy of the FEC was not important in the re-used samples (as a FEC had already been performed on them when they were fresh, to

inform the owners) this was not thought to be important. Egg counts have been shown to drop with sample storage (Sengupta *et al.*, 2016), however the G1 control values for the validation had been prepared on the same day as the G2 results were collected, so the phenomenon was not merely due to eggs having hatched. It is possible that the sedimentation process was affected once the samples became older, and further work to test samples repeatedly on consecutive days could pin-point the maximum sample age for which the G2 protocol should be used. However, subsequent work on the quality of images produced meant that improvements were planned in the imaging device, rendering such work redundant until the improved device became available. To the best of the author's knowledge no work has yet been completed on the age of eggs and the ability to sediment.

As the purpose of the FECPAK^{G2} is to enable on-site imaging of the prepared samples, it was decided that there was no reason that imaging of samples over a week old would need to be undertaken, so this phenomenon was not considered to affect the validation. In fact, the ability of the FECPAK^{G2} system to enable FECs to be undertaken by the owner on fresh samples is an important development, especially as the recommendation for equine faecal samples is that they be tested within four days of collection (Sengupta *et al.*, 2016).

3.5.1.2 Effect of height of animals on G2 performance

Other potential sources of variation in the G2 test's relative accuracy were considered, including the size of the animals that provided the faecal samples. The re-used samples had come from a rescue centre that had a lot of small ponies, and the next set of samples that were tested had come from a showing yard that also had

small ponies. Due to this concern, the results were re-analysed taking into account the size of the animals concerned.

One idea which had been considered as a possible contributor to the poorer results of the G2 method for smaller animals was a difference in the constituents of the faeces. Faeces are not merely composed of indigestible food material, but also contain intestinal mucus and sloughed off intestinal cells (Tortora and Anagnostakos, 1987). It was postulated that the smaller intestinal diameter of smaller animals may have led to a larger proportion of these lipid products due to the higher surface area to volume ratio of a smaller diameter intestine. Lipid floating on the meniscus in the Micro-I cassette could quite possibly affect the accumulation of eggs due to differing surface tension (Yun Xu *et al*., 2013), and therefore lead to poor performance of the G2 test. It was hoped to analyse the constituents of poorly performing samples from smaller animals and compare them to analyses of samples from larger animals that had performed well in the G2 test.

When examining the dataset as a whole there was a correlation between the height of animal and the accuracy of the G2 test compared to the G1 control. When the data were split into animals over 15 hh or under 15 hh, the percentage accuracy of the G2 test compared to the G1 controls dropped from 101% in the animals over 15 hh to 75% in the animals under 15 hh. However, later analysis pinpointed the drop in comparative accuracy of the G2 test to the introduction of the scoop measure, so the effect of height may have been a random artefact. Future work analysing the faecal composition of different sizes of horse and pony and how this affects egg accumulation on the meniscus of a flotation solution could be performed to investigate this potential effect further.

3.5.1.3 Change in protocol – scoop measure vs sedimentor measure

Although the faecal scoop had been observed to provide an improved protocol during the optimisation from the comparative ease of scooping a level measure of slurry rather than spooning it into the sedimentor up to the slurry line, the results produced by using the scoop were poor when compared to the G2 protocol that spooned the faecal slurry into the sedimentor up to the slurry line. This was, perhaps, unsurprising in retrospect due to the fact that the sedimentors had an average volume of 13 ml at the 12 ml mark. The samples measured using the sedimentor returned a mean G2 relative accuracy of 96% of the G1control counts, whereas those measured using the scoop had a mean relative accuracy of 68% of the G1 controls. After the imaging problems identified in Section 3.4.3 were highlighted as the cause of the poor performance of the G2 test, it was understood that the increased volume of faecal slurry that was used when measuring with the sedimentor was compensating for the eggs lost during the imaging process.

It was decided at this point that the G2 protocol should measure the slurry using the sedimentor, as this was standard operating practice for the sheep protocol. As Techion set the multiplier for the G2 (equine test) sensitivity to 45 epg this gave a mean relative accuracy of 101% for the sedimentor measured samples (the calculated multiplier is 43 epg, which has been used to produce the results in this thesis).

As the point of the validation work was to demonstrate that the FECPAK^{G2} protocol produced FECs comparable with the G1 control values when used as directed, measuring the slurry using the sedimentor as for the sheep protocol was seen as reasonable. When the egg loss due to problems with the imaging software were highlighted this provided a potential reason that the multiplier needed to be increased

in order to preserve relative accuracy, and also why the samples measured using the sedimentor, which had a tendency to include more slurry, performed better than those measured using the scoop. Once a device with improved imaging capabilities is available, repeating this validation work would identify if a scoop measure could again be used for greater relative accuracy.

3.5.2 Validation 20 ml protocol

In all equine FECs performed, using the G1, G2 Slide or G2 Cassette, the G2 Slide preparations produced higher FECs than the G1 control counts. However, when this same preparation was imaged using a G2 cassette and the Micro-I, the FEC epg values were significantly lower – lower, in fact, than those from the controls.

Given that the G2 preparation was imaged through a slide and the Micro-I it is clear that the preparation itself is capturing helminth eggs evidenced by the G2 Slide counts which were consistently higher than the G1 control values. With the G2 preparation capturing helminth eggs it is likely that egg losses observed between the slide counts and the Micro-I cassettes were being lost at the point of imaging within the cassette. The FECs produced from the G2 cassettes were consistently lower than those from counting the same preparation using a slide and consistently lower than the control results from the G1 protocol.

When the imaging process was investigated (Section 3.3.4) it was discovered that the stacking software was sometimes choosing out of focus images to produce the final stacked image that would be used to identify the helminth eggs. This effect was greater where more eggs were present, as a blurry image portion would have the potential to hide a greater number of eggs and therefore render the G2 less accurate. Because of the greater volume of faecal slurry used in the 20 ml protocol compared

to the 12 ml protocol, a greater number of eggs would be expected for a given infection level, increasing the likelihood that eggs would be hidden. This led to a comparatively less accurate test when the 20 ml protocol was imaged in the Micro-I compared to the 12 ml protocol.

Despite the challenges with the 20 ml protocol this process was not in vain. The 20 ml G2 protocol counted with a slide rather than the Micro-I produced consistently higher FECs than the G1 control values. In addition the G2 Slides were exceptionally clear and easy to read as there was barely any debris visible when 1 ml was spread out over the surface of a slide rather than accumulating within a 3 mm circle. Therefore, this protocol (G2 Slides) would likely be useful in the future resistance testing work where a more sensitive test increases accuracy (Levecke *et al.*, 2011).

In addition, this work on image analysis has identified the source of the problem as a software issue resulting in eggs that were actually visible to the Micro-I being missed in the final stacked image produced. Once an improved imaging platform was available, it would be worthwhile to repeat this validation of the 20 ml protocol to produce a more sensitive FECPAK^{G2} test.

3.5.3 Image assessment

The liquid in the FECPAK^{G2} cassette forms a meniscus with a domed shape (Figure 3-10). In order to form a focused, two-dimensional image of the surface of this meniscus, four separate images are taken at different heights. These images are computationally "stitched" together to form a stacked image. Thus, the stacked image is formed from four concentric circles, one from each image, such that each circle represents the portion of that individual image that is in focus.

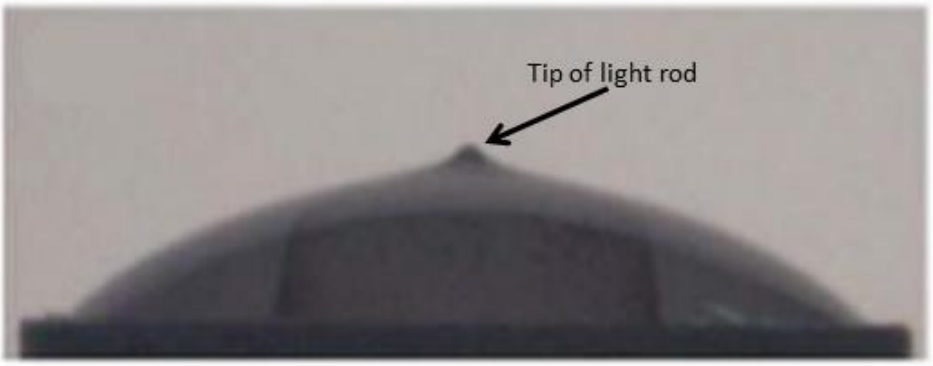

Figure 3-10 Meniscus formed in FECPAK^{G2} imaging cassette (Cooke *et al*., 2016).The tip of light rod is highlighted. The light rod forms the 3 mm field of view visible on the final image captured by the Micro-I and where helminth eggs accumulate.

When the G2 cassettes were taken out of the Micro-I and re-inserted again, the contained liquid swirled around to a certain extent, meaning that the images looked quite different. With the timed capture software, the G2 cassettes were not moved in between images. Thus, the debris etc. remained in the same place on each repeat image. This provided an easier approach to determine whether the eggs were being missed rather than merely having moved to a different part of the well.

It became apparent from the repeat imaging work that the stitching software was not functioning correctly, and it was selecting some poorly focused rings to form the final stack. This led to egg counts being understated and variable, as visible eggs were not all present in the stacked images. This information was presented to the industry partner, Techion NZ, who then performed their own experiments and confirmed that the issue also occurred in their laboratory.

In the interim, while software improvements are being made to counteract this issue, it was decided that all imaging should be processed by using the timed-capture software. This would enable any experimental protocols to be evaluated in the

knowledge that any perceived under-performance was real and not merely eggs being missed by focus issues.

After having re-examined the existing data that compared the same preparations on both G2 Slide and G2 Cassette, it was discovered that the more sensitive 20 ml protocol actually did not perform any worse than the 12 ml protocol that was decided on as the final version. It was decided that once the imaging issues were solved, it would be worth re-visiting the validation of the 20 ml G2 Cassette protocol. Likely the improved egg recovery of the G2 protocol compensated for the deficiencies in the imaging process, and that even though the 12 ml G2 Cassette protocol performed as well as the G1 controls, the potential exists to produce an improved G2 Cassette test using the 20 ml protocol once the imaging device has been improved.

3.5.4 Statistical analysis

The significant positive correlation between the mean G2 results and the G1 controls was independently true for both the UK and the NZ data, therefore we can be confident that the consistency between G1 and G2 methods was independent of the laboratory in which they were tested. Egg count per sample was equally consistent between the G1 and G2 methods whether looking at the UK data, the NZ data or the combined dataset. This illustrates that the G2 method worked consistently regardless of the location of sampling or the operative performing the test. Importantly, relative accuracy of the G2 method did not significantly change with rising FEC levels. The samples tested ranged from a FEC of 100 epg to over 3,000 epg. Therefore, the G2 test was found to be accurate over a wide range of FEC levels. Given that the G2 test is designed as a diagnostic aid to determine whether anthelmintic treatment is

required, and with the accepted threshold for treatment at 200 epg (Coles, 2009), improved accuracy at very low infection levels (below 90 epg) is not required.

3.6 CONCLUSION

After the imaging issues seen were reported to Techion, company led investigation into additional laboratories using the Micro-I and G2 cassette (in New Zealand and Belgium) confirmed that it was not an isolated occurrence. As these focus issues were not dependent on the protocol being used to prepare the sample, there remains a good chance that, once these difficulties are rectified, more sensitive protocols could be imaged successfully in the G2 cassette.

It had previously appeared that the more sensitive protocols were less effective at producing results in the G2 cassette. In fact, inaccuracies were caused by eggs being missed by the focussing software, rather than by the eggs failing to accumulate into the visible field of view. Where more eggs were expected (such as in a more sensitive protocol), there was a greater chance that eggs would be missed by focusing issues. After this effect was identified, it was decided that there was the potential for an ultra-sensitive protocol to be developed, once the focus issues were solved. In the meantime, the timed-capture software would be used to capture the images, in order to mitigate the focus problems as much as possible.

Thus, the aim of this work was to compare the G2 system to the existing G1 method of faecal egg counting and prove that it performed as well as, or better than, the existing validated method. It was noted that the FECPAK^{G2} system represented an improved alternative to conventional slide based faecal egg counting for horses, producing comparable results, to the FECPAK^{G1} counterpart. As the FECPAK^{G2}

methodology does not require faecal samples to be sent by post or taken to a testing laboratory as with other commercially available methods (https://www.westgatelabs.co.uk/, https://www.poopost.co.uk/, https://www.efecs.co.uk/), it represents a more convenient method of performing FECs than others available. In addition, the fact that experienced technicians are responsible for identifying the helminth eggs increases accuracy compared to wholly owner-performed FECs which have been found to over-estimate egg counts (McCoy *et al.*, 2005). It is hoped that the availability of the FECPAK^{G2} method will encourage horse owners to perform FECs for targeted selective treatments (Nielsen *et al.*, 2014a) and that this will promote the longevity and sustainability of anthelmintics for controlling nematode parasites of horses.

Horse owners intending to use FECs as part of their deworming strategy were more confident that they would effectively control helminth parasites in their horses, as evidenced by (Rose Vineer *et al.*, 2017). This suggests that owners having the ability to perform their own FECs and have them analysed by experts, would have a significant, positive effect on the uptake of targeted treatment strategies for worm control in horses.

4 MONITORING THE EQUINE NEMABIOME IN RESPONSE TO ANTHELMINTIC TREATMENT

4.1 INTRODUCTION

New molecular tests termed nemabiome barcoding are now available to support comprehensive parasitic nematode diagnostics by both quantifying and determining the relative proportions of species in nematode communities infecting a host animal. Nemabiome profiling was first developed for gastro-intestinal nematode (GIN) communities of cattle, by culturing nematode larvae from the eggs in the host faeces and undertaking deep amplicon Next Generation Sequencing (metabarcoding) of internal transcribed spacer 2 (ITS-2) rDNA (Avramenko *et al.*, 2015). Recent research in equines has realised the possibility of performing nemabiome sequencing directly on faecal samples via ITS-2 rDNA locus analyses, thus saving the time consuming larval culture step (Mitchell *et al.*, 2016). Nemabiome sequencing has been demonstrated to be equally effective whether sequencing DNA taken from nematode eggs or from larval cultures in ovine samples (Redman *et al.*, 2019). Furthermore, molecular genetic techniques have been proposed for measuring the change in the nemabiome following anthelmintic treatment in cattle using this same deep amplicon sequencing approach (Avramenko *et al.*, 2017).

Thus, next generation sequencing (NGS) platforms such as Illumina (Meyer and Kircher, 2010), SOLiD (Hedges *et al.*, 2011) and the MinION (http://www.nanoporetech.com/) offer new potential for discovery and applied parasite research (Wit and Gilleard, 2017). For example, The Oxford Nanopore MinION offers real time direct sequencing of native or amplified DNA / RNA at low cost and can read any length strand of nucleic acid, making it a useful tool for helminth nemabiome sequencing in relation to anthelmintic exposure. In brief, Nanopore technology monitors changes in electrical current as nucleic acids are

passed through a protein "nanopore" (Figure 4-1; http://www.nanoporetech.com/). With nanopore sequencing both 1D and 2D reads, either one or both of the complementary strands of the DNA, are possible (Jain *et al.*, 2016). The genetic sequences produced from such a sequencing approach can be compared against known sequences for the different nematode species, enabling a non-invasive and relatively easy method of determining which species are infecting an individual horse.

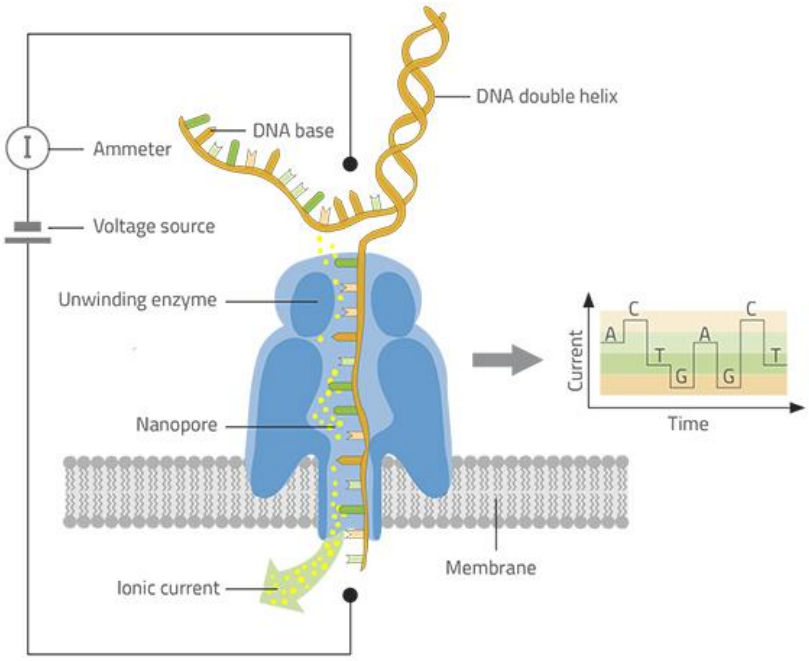

Figure 4-1 Pictorial representation of Nanopore sequencing, showing the ID read of a DNA strand through a protein nanopore. Image from (Göpfrich and Judge, 2018).

In order to acquire pre- and post-treatment faecal samples for nemabiome genetic analysis, it was first necessary to identify horses which harboured anthelmintic resistant helminth parasites. Post-treatment faecal samples from horses with drug anthelmintic resistant parasites would still contain helminth eggs, and enable a comparison to be made between the nemabiomes before and after treatment. FECs

were performed on these faecal samples by counting multiple slides to reduce the multiplication factor used to either 4.6 (standard resistance testing protocol) or 6.25 (four FECPAK[G1] slides) This is important as when performing Faecal Egg Count Reduction Tests (FECRTs) a higher sensitivity test results in improved accuracy (Levecke *et al.*, 2011).

In order to determine the nemabiome of the horses that provided the samples in the resistance testing section (Section 4.3.1) genetic sequencing of the samples collected both pre- and post-treatment, would be performed to determine the species composition. In order to identify the species of parasite contained in the faecal the samples, DNA would be extracted and then a section amplified using Polymerase Chain Reaction (PCR). This would provide a number of sequences unique to individual nematode species which could then be compared to a database of known sequences, and the proportions of each species identified calculated. The samples would also be tested to determine if Single Nucleotide Polymorphisms (SNPs) were present that correlated with BZ resistance.

4.2 CHAPTER AIMS

- Monitor anthelmintic resistance to different anthelmintic classes in several yards across the UK
- Collect pre- and post-treatment faecal samples where anthelmintic resistance is identified
- Identify primers from the literature that could be used to identify cyathostomin species and identify SNPs associated with BZ resistance

- Optimise DNA extraction procedure from faecal samples
- Investigate the possibility of extracting helminth DNA from FECPAK^{G2} preparations
- Extract helminth DNA from the faecal samples and amplify using PCR
- Sequence the helminth DNA using a MinION benchtop sequencer (https://nanoporetech.com/) and track changes in the nemabiome in response to anthelmintic treatment

4.3 MATERIALS AND METHODS

4.3.1 Nematode Resistance monitoring pre and post anthelmintic treatment

In total, fifty faecal samples were taken from horses across two yards in West Wales during the summer of 2016, 25 per yard. From each sample from the first yard, two G1 preparations were made, according to the standard FECPAK^{G1} testing protocol described in Section 7.1 "Standard FECPAK G1 protocol". Three slides were counted from the first preparation, and one slide counted from the second preparation. The samples were processed using the FECPAK^{G1} method in order that the data could be used as part of the validation of the FECPAK^{G2} system. From the second yard, samples from each horse were prepared using the resistance testing protocol developed during the optimisation phase and described in Section 7.2 of the appendix, giving a sensitivity of 4.6 epg.

All horses with a pre-treatment FEC of 200 epg or over (the suggested treatment threshold in Coles (2009)) were treated with Ivermectin paste as per the manufacturer's instructions, owner-administered, and post-treatment samples

collected on day 14 after treatment. Post treatment samples were all tested using the standard resistance testing protocol (section 7.2 of the appendix).

During the summer of 2017, faecal samples were taken from 67 horses in three yards in the UK. The yards used were the two yards from the 2016 study of Ivermectin resistance, plus one yard in North East England. Pre-treatment samples were tested using the resistance testing protocol described in section 7.2 of the appendix.

All horses with a pre-treatment FEC of 200 epg or over were treated with liquid Fenbendazole (Panacur 10% suspension) at a rate of 1 ml per 13 kg bodyweight per manufacturer's instructions. Anthelmintic was owner-administered, in feed. Post treatment samples were collected on day 14 after treatment, and tested using the same resistance testing protocol.

4.3.2 Molecular genetics

4.3.3 Primer selection: rDNA

In order to determine which nematode species were present within faecal samples, a portion of the small sub-unit of the ribosomal gene (see Figure 4-2) would be amplified using PCR. This gene is highly conserved among nematodes, and as the MinION is a powerful sequencer, it was decided to amplify the largest possible segment of this gene. The aim of this section of work was to select primers that had a forward primer as close to the 5' end of the 18S region, and reverse primers that were as close to the 3' end of the 28S region as possible.

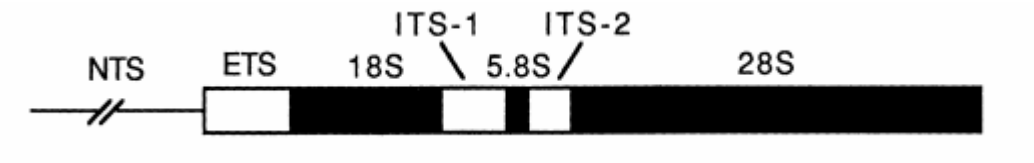

Figure 4-2 Schematic diagram of ribosomal gene of eukaryotic DNA from (Hillis and Dixon, 1991) NTS = Non-Transcribed Spacer; ETS = External Transcribed Spacer; ITS = Internal Transcribed Spacer; 18S, 5.8S and 28S = genes

From the literature, a number of papers were selected which used PCR to amplify the a portion of the rDNA gene from nematodes (Table 4-1)

Table 4-1 Primers to amplify rDNA taken from the literature

Paper	Forward primer	Reverse primer	Product
Powers *et al.*, (1997)	5'-TTGATTACGTCCCTGCCCTTT-3' located in the 3' portion of 18S, the small ribosomal subunit gene, approximately 190 bp from its junction with ITS1, the first internally transcribed spacer.	5'-GTAGGTGAACCTGCAGATGGAT-3' located in the 5' portion of 28 S, the large ribosomal subunit gene, approximately 80 bp from the junction with ITS2, the second internal transcribed spacer	Between both spacers is the 5.8 S ribosomal gene that is generally around 155 bp in length
Peachey *et al.*, (2017)	5′ GATTGATTCTGTCAGCGCTATA 3′	5′ TAATGAGCCGTTCGCAGT 3’	Giving 99 bp housekeeping gene, the 18S rRNA gene
Marek *et al.*, (2010)	5’-TTGATTAGGTCCCTGCCCTTT-3’	5’-TTTCACTCGCCGTTACTAAGG-3’	Gives the region of the ribosomal DNA cistron including the 3’end of the 18S gene, ITS1, 5.8S, ITS2 and the 5’ end of the 28S gene
Floyd *et al.*, (2005)	5’-CGCGAATRGCTCATTACAACAGC-3’	5’-GGGCGGTATCTGATCGCC-3’	The primers amplify an internal fragment of the 18S, approximately 900 bp in length (the full-length gene is approximately 1700 bp): the forward primer binds at a site around 100 bp inward from the 5′end of the gene, and the reverse primer at around 700 bp inward from the 3′ end. This gives nematode specific 18S DNA and avoids contamination from fungi.
Mitchell *et al.*, (2019) Avramenko *et al.*,(2015)	’-ACGTCTGGTTCAGGGTTGTT-3’	5’-TTAGTTTCTTTTCCTCCGCT-3’	Gives ITS2 region. Taken from Gasser *et al* (1993). This gives a segment 250 bp long, and was used by Mitchell to speciate cyathostomins in equine faecal samples

4.3.4 DNA Extraction QiAgen kit

4.3.4.1 DNA Extraction QiAgen kit with beads

DNA extraction from equine faeces was performed with a QiAmp DNA stool kit (QiAgen.com). The standard protocol was modified to include a bead disruption step. A sample of 350 mg of frozen faeces were added to a 2 ml micro-centrifuge tube, along with 1.6 ml Buffer ASL and four 1mm Thistle zirconia / silica beads (ThistleScientific.co.uk). The tube was vortexed horizontally for two minutes, then incubated at 95°C for five minutes, inverting halfway through to ensure even heating. The manufacturer's recommended protocol was then followed.

The DNA extraction was repeated using a Tissue Lyser (QiAgen, Hilden, Germany). This time, 250 mg of frozen faeces were placed in a 2 ml tube, along with one 1.5 mm glass bead, and 2 ml of buffer ASL. The samples were bead-beated in a Tissue Lyser (QiAgen, Hilden, Germany) at 50 beats per second, for three minutes. Two ml of the lysate was transferred into a new 2 ml tube, and the protocol continued as before.

4.3.4.2 DNA Extraction QiAgen kit without beads

Faecal DNA was also extracted using the QiAmp standard mini-stool kit protocol without the addition of beads. The protocol followed was identical to that in the first part of section 4.3.4.1 with the exception that in the initial step, 260 mg frozen faeces and 2 ml of Buffer ASL were added to the 2 ml micro-centrifuge tube, with no Thistle beads.

4.3.4.3 DNA Extraction QiAgen kit freeze drying

In order to discover whether a greater amount of starting material could be used for the DNA extraction, freeze drying prior to DNA extraction was also assessed. In

brief, 5 g of faeces was freeze-dried for four days, which reduced the mass of each sample to around 1.5 g each (range 0.9 g to 2.9 g). This freeze dried material was then compared to the frozen samples with and without beads using the QiAmp standard mini-stool kit following the manufacturer's instructions with minor modifications as stated in sections 4.3.4.1 and 4.3.4.2 .

4.3.4.4 DNA extraction from Ascarid tissue and Panagrellus redivivus using QiAgen kit

In order to obtain DNA to use as a positive control, an adult female Ascarid was obtained from a colleague, and DNA extracted using a QiAmp Blood and Tissue kit (h ttps://www.qiagen.com/us/). Fourteen mg of Ascarid tissue was chopped and placed in a 1.5 ml Eppendorf and the protocol followed as per the manufacturer's instructions.

In addition, DNA was extracted from *Panagrellus redivivus,* a free living nematode which is maintained in-house at Aberystwyth University. A mix-stage active culture of *P. redivivus* on autoclaved porridge oats (Papadopoulos *et al.*, 1989) was sourced from stocks , and distilled water was gently floated onto the culture surface in order to decant via pipette nematodes into a 1.5 ml Eppendorf. The nematodes were subsequently allowed to settle under gravity, and the supernatant drawn off in order to remove any residual floating porridge sediment and live nematode presence confirmed by low power light microscopy. DNA extraction was performed using a QiAmp blood and tissue kit (https://www.qiagen.com/us/) following the manufacturer's standard protocol.

4.3.5 DNA Extraction: CTAB (Cetyl Trimethylammonium Bromide) method

Following the difficulties experienced in optimising DNA isolation using QiAgen kits, extraction of DNA from equine faecal samples using the CTAB method was attempted. The CTAB protocol itself was adapted from Dellaporta *et al.* (1983), Yu and Morrison (2004) and William *et al*. (2012).

In brief, lysis buffer was prepared containing 50 mM EDTA pH 8, 500 mM Tris HCL pH8 and 500 mM NaCl. This was autoclaved at 121ºC for 15 minutes, and 4% (w/v) SDS was added while it was still warm, and stirred until it dissolved. The CTAB/NaCl was prepared, containing 700 m NaCl and 10% (w/v) CTAB, and autoclaved.

Initially, a comparison between freeze-dried and frozen faecal sample was completed, following earlier experimental work using freeze-dried samples. This work had discovered that freeze-drying merely reduced the weight but not the volume of the sample, and therefore did not permit a greater quantity of sample to be processed, but as the CTAB protocol stipulated freeze-dried material, this was revisited.

Two 2 ml tubes were prepared, with equal volumes of sample which equated to 50 mg of freeze-dried sample or 200 mg frozen sample. The CTAB method was followed as described in Section 7.3 of the Appendix.

In common with the findings from Section 4.3.4.2 (DNA Extraction QiAgen kit without beads), it was decided that there was no benefit in freeze-drying the samples before DNA was extracted, and so approximately 200 mg of frozen sample was used for each extraction, and the CTAB protocol followed as above. Ten samples were

prepared simultaneously each time, and the work was repeated until DNA had been extracted from all the samples.

4.3.6 DNA Extraction from FECPAK^{G2} Cassette preparations

A 1.5 ml aliquot of a FECPAK^{G2} preparation that had been processed for faecal egg counting and stored at -20°C was used for DNA extraction. When counted, the faecal preparation had shown 17 eggs in 0.5 ml, so the sample potentially contained approximately 51 nematode eggs. In order to separate the eggs from the saline solution, a number of wash steps were performed. The sample was diluted with 10 ml distilled water, and centrifuged for 5 minutes at 276 × *g* and 5 minutes at 1500 × *g*, the supernatant discarded and the pellet re-suspended in 1 ml distilled water. This solution was transferred into a 1.5 ml micro-centrifuge tube and centrifuged at 22,000 × *g* for 5 minutes, the supernatant discarded and the pellet re-suspended in 200 µl distilled water. The protocol for the DNeasy Blood and Tissue kit (https://www.qiagen.com/us/) was then followed according to the manufacturer's instructions.

Extracted DNA was quantified using a Nanodrop 1000 spectrophotometer (http://www.thermofisher.com).

In addition, 500 µl of a FECPAK^{G2} Cassette preparation was used, that had been stored at -20°C. From the egg counting data, this sample potentially contained between 9 and 34 eggs. The sample was warmed to room temperature, and the protocol above followed, omitting wash steps. The DNA was again quantified using a NanoDrop 1000 spectrophotometer (http://www.thermofisher.com).

The DNA extraction from the unwashed sample was also unsuccessful, so it was decided to experiment with different preparation methods at the faecal sample stage, to see if the salt was potentially causing an issue with the extraction, or if washing the sample was losing the eggs, or the eggs were deteriorating due to low temperature storage. In order to do this, a fresh faecal sample was obtained (FECPAK^{G2} slide count FEC quantified this sample at 184 epg) and was prepared using the 20 ml FECPAK^{G2} protocol described in Appendix 1 "Standard Resistance testing protocol". A second sedimentor was prepared, but instead of diluting the sediment with 80 ml saline, 80 ml water was used. Three 1 ml aliquots were taken, one from the regular protocol, and two from the water-diluted sedimentor. One of these water-diluted aliquots was centrifuged at 22,000 × *g* for three minutes and the supernatant discarded. The DNeasy protocol above was followed on all three aliquots.

Following the success of the CTAB DNA extraction method on the faecal samples, this method was also attempted on the FECPAK^{G2} cassette preparations.

Two 1 ml aliquots of FECPAK^{G2} cassette preparation were selected, which had been stored at minus 20ºC. The samples had FECs of 478 epg and 874 epg, meaning that the samples would be expected to contain 21 and 38 eggs respectively.

The standard CTAB DNA extraction protocol was performed (see Section 7.3 of the appendix), with the exception that in step four the tubes were centrifuged for three minutes at 500 × *g* and one minute at 900 × *g*, to ensure that all eggs were recovered.

4.3.7 PCR optimisation rDNA

In order to determine the species of nematodes in the faecal samples, optimisation of primers and PCR conditions for amplifying a segment of the ribosomal gene was required. Primers were selected from the literature, as described in section 4.3.3 Primer selection: rDNA.

All primers identified in the literature were initially tested. This was performed using a sample of DNA extracted using the QiAmp kit without bead disruption with a positive control of *Biomphalaria glabrata* DNA (donated by Dr K. Geyer). For PCR, 25 µl reactions were performed using MyTaqRed DNA polymerase (Bioline, UK) according to the manufacturer's instructions with 10 µM forward and reverse primers and approximately 100 ng of template DNA.

PCR conditions were: initial denaturation at 95ºC for 1 minute, denaturation at 95ºC for 15 seconds, annealing at 55ºC for 15 seconds, extension at 72ºC for 10 seconds and final extension at 72ºC for 5 minutes, with 35 cycles performed.

A second DNA sample was tested which had been extracted using the QiAgen kit vortexed for 2 minutes with 4 × 1mm Thistle beads. Primers tested were 'Floyd', 'Marek' and 'Peachey'. As the DNA was of a lower concentration it was first concentrated using a speed vac (Concentrator plus, Eppendorf UK) for 20 minutes.

PCR conditions were slightly altered, reducing the annealing temperature to 53 ºC to see if this would strengthen the band produced by the Peachey primers and my extracted DNA sample. The number of cycles was increased to 40 to maximise the amount of DNA amplified.

The ‘Floyd’, ‘Marek’ and ‘Peachey’ primers were tested again, in attempt to find better positive controls, which were ascarid DNA and Schistosomule DNA. As the product from the ‘Peachey’ primers was only 99 bp it was considered to be too small, and so the primers from ‘Floyd’, ‘Marek’ and ‘Powers’ were again tested using ascarid, Schistosomule and *P, redivivus* DNA with the PCR annealing temperature increased to 56ºC.

All PCR products were imaged on a 1% agarose gel at 100 V using SYBR safe DNA gel stain (https://www.thermofisher.com, to check that a band of the expected size was generated.

4.3.8 PCR of DNA extracted using QiAgen kit

The aim of this work was to amplify a region of nematode DNA from the faecal samples, using the extraction conditions and the primer set that were considered to perform the best. Once the ‘Floyd’ primers had been selected as the most promising ones to use, and a dozen faecal samples processed using the optimal method of extraction (frozen samples, without a bead disruption step), the samples were tested using PCR. PCR conditions were as before using the ‘Floyd’ primers.

PCR products were imaged on a 1% agarose gel at 100 V, to check that a band of the expected size was generated.

4.3.9 PCR of DNA extracted using CTAB method

The ‘Floyd’ primers were selected, as these were the most promising to produce a long fragment of the rDNA gene, and were tested on ten of the DNA samples extracted using the CTAB method using the PCR conditions previously optimised. The PCR from the above experiment failed to amplify anything from the ten samples

extracted using the CTAB method, so the experiment was repeated using only approximately 20 ng of template.

As there had been success for a couple of the samples using 20 ng of template, it was decided to try the primers used in Mitchell *et al*. (2019), also Avramenko *et al.* (2015) which had been taken from Gasser *et al*.(1993), using 20 ng of template DNA. These primers gave a smaller expected product size of 250 bp, but had successfully been used to identify cyathostomin species in Mitchell *et al*. (2019). PCR annealing temperature was reduced to 55ºC.

PCR products were imaged on a 1% agarose gel at 100 V, to check that a band of the expected size was generated.

4.3.10 Primer selection: β tubulin isotype 1

Literature was examined for primers which would amplify either the entire β tubulin gene, or those SNPs associated with BZ resistance in cyathostomins. The results are shown in Table 4-2.

It was decided to use the primers from Hodgkinson *et al.* (2008), as they amplify the whole of the β tublin isotype 1 coding sequence, and also to assess the primers from (Ishii *et al.* (2017) in the event that the 'Hodgkinson' primers were unsuccessful. The third set of primers, from Coles *et al.* (2006) and Pape *et al.* (2003) were not used, as they were designed to amplify separately susceptible and resistant β tubulin isotypes, given this would not be necessary when PCR products would be sequenced using the MinION.

Table 4-2 Primers to amplify all or part of the β tubulin gene in GI nematodes taken from the literature.

Paper	Forward primer	Reverse primer	Notes
Coles *et al.*, (2006)	5'-GGTTGAAAATACAGACGAGACTTT-3' (susceptible allele) 5'-GGTTGAAATACAGACGAGACTTA-3' (resistant allele)	5'-AGCAGAGAGGGGAGCAAAGCCAGG-3' (same for both alleles)	
Pape *et al.*, (2003)	5'-GGTTGAAAATACAGACGAGACTTT-3' (susceptible allele) 5'-GGTTGAAATACAGACGAGACTTA-3' (resistant allele)	5'-AGCAGAGAGGGGAGCAAAGCCAGG-3' (same for both alleles)	Cn24FS (first forward primer) is able to detect the base associated with phenylalanine in codon 200, whereas Cn25FR (second forward primer) detects tyrosine at this position. Thereby only the final base at the 3' end of each forward primer, corresponding to the middle base of amino acid 200, confers the specificity.
Hodgkinson *et al.*, (2008)	5'-AACGCAATCAATGTGTATTTCGC-3'	5'-GGTTTAATTACCCAAGTTTGAG-3'	These amplify isotype 1 of the β tubulin gene. They amplify the full length gene. The one listed here as the reverse primer was called the "nematode splice leader"
Ishii *et al.*, (2017)	5′-GCTAACTCACTCACTTGGAGGA-3′	5′-CTTTGGTGAGGGAACAACG-3′	These amplify codon 167 and give a fragment 120 bp in length

4.3.11 PCR optimisation beta tubulin

The β tubulin gene is the drug target for the benzimidazole class of drugs (Lacey, 1989). Sequencing this gene would enable identification of mutations in the gene that might either be existing known mutations, or potential novel ones.

The first primer set to be tested were those from Hodgkinson *et al.*(2008), which amplify the full length gene of isotype 1 of the β tubulin gene. Once again, the diluted CTAB DNA was used as the sample template (20 ng per reaction) and *P. redivivus* DNA used for the positive control. For PCR, 25 µl reactions were performed using MyTaqRed DNA polymerase (Bioline, UK) according to the manufacturer's instructions with 10 µM forward and reverse primers and approximately 20 ng of template DNA.

PCR conditions were as for the rDNA work, with an annealing temperature of 63ºC. In the paper that the primers were taken from (Hodgkinson *et al.*, 2008), RNA was extracted from adult worms and used to make cDNA. The PCR amplified the whole of isotype 1 of the beta tubulin gene, and made a product of 1437 bp. Because in this project, gDNA was being used, the product would possibly be even larger than 1437 bp, and so it was decided to revisit the PCR conditions to give a longer extension time, to facilitate the amplification of a large product. It was also decided to use increase the quantity of template DNA to 80 ng. PCR conditions – changed as per Hodgkinson *et al.* (2008) to initial denaturation at 94ºC for 1 minute, denaturation at 94ºC for 1 minute, annealing at 63ºC for 1 minute, extension at 72ºC for 1 minute and final extension at 72ºC for 10 minutes, with 35 cycles.

As the above experiment still did not amplify anything, the experiment was repeated twice more. The first repeat had the extension time increased to two minutes per cycle, and the second time it was increased to three minutes per cycle.

Having failed to amplify the whole beta tubulin gene, it was decided to try the primers from Ishii *et al*.(2017) which amplify only codon 167, one of the two codons associated with BZ resistance in cyathostomins (von Samson-Himmelstjerna *et al.*, 2007a). As these primers would give a smaller product they had been considered inferior to the 'Hodgkinson' primers, but would possibly be easier to amplify. For PCR, 25 µl reactions were performed using MyTaqRed DNA polymerase (Bioline, UK) according to the manufacturer's instructions with 10 µM forward and reverse primers and approximately 20 ng of template DNA and PCR conditions of: initial denaturation at 95ºC for 1 minute, denaturation at 95ºC for 15 seconds, annealing at 53ºC for 15 seconds, extension at 72ºC for 10 seconds and final extension at 72ºC for 5 minutes, with 40 cycles.

All PCR products were imaged on a 1% agarose gel at 100 V, to check that a band of the expected size was generated.

4.3.12 Cloning and sequencing nematode DNA

In order to check that the PCR was correctly amplifying nematode DNA, prior to preparing and sequencing PCR products using the MinION (https://nanoporetech.com/), PCR products were inserted into the PGemTeasy sequencing vector (Promega, UK) and cloned using *Escherichia coli*. Each bacterium would then likely contain a single rDNA insert (rather than the mixed population amplified from the faecal DNA samples), which could be sequenced using Sanger sequencing to determine the species from which it came.

4.3.12.1 Cloning and sequencing, High Fidelity PCR

Hi fidelity PCR was performed using MyFi 2x mix polymerase (Bioline UK) with 25 µl reactions according to the manufacturer's instructions with 10 µM forward and reverse primers and using approximately 20 ng of template DNA. PCR conditions were as in section 4.3.3 Primer selection: rDNA with an annealing temperature of 55ºC and 25 cycles used to maximise accuracy.

For this experiment, two positive controls (*P. redivivus* and *Baylisascaris schroederi*) were used, and two negative controls (no DNA template, and DNA from a faecal sample with a zero FEC).

Ten µl of each PCR product along with 2.5 µl of loading dye were run on a 1% agarose gel at 100 V, to check that a band of the expected size was generated. The two amplicons selected for transformation into *E. coli* were Widget pre and Major pre.

4.3.12.2 PCR product purification, ligation and cloning

PCR products from the selected samples were purified using an Invitrogen Pure Link Quick Gel Extraction and PCR Purification Combo kit (https://www.thermofisher.com) to remove primers, dNTPs, enzymes, short-failed PCR products and salts from the PCR products following the manufacturer's instructions.

The purified PCR products were then ligated into the PGEM-T Easy vector (www.promega.co.uk) according to the manufacturer's instructions using 3 µl of the purified PCR products.

The vector was then inserted into Alpha select Bronze Efficiency *E. coli* cells (https://www.bioline.com/uk/), which contain a lacZ marker that provides α-complementation of the ß-galactosidase gene for blue/white colour screening, enabling visual confirmation that the plasmid has been successfully inserted in the colony selected for sequencing. Transformation was performed as per the manufacturers' guidelines with heat shock at 42°C for 30 secs before ice for 2 mins. Transformed cells were revived with SOC media (www.sigmaaldrich.com) and the tubes placed into the shaking incubator at 37ºC 200 rpm for ninety minutes. Transformed E. coli was then plated out on IAX plates (containing 3.5% w/v LB premix (Miller brand, www.fishersci.co.uk) and 0.1% X-GAL in DMF (5-Bromo-4-Choro-3-Indolyl-β-D-Galactoside, Melford Laboratories Ltd, Ipswich), 0.1% IPTG (Isopropyl-β-D-thiogalactopyranoside, www.fishersci.co.uk) and 0.1% Ampicillin (www.fishersci.co.uk)).

Following overnight incubation at 37ºC, the plates were examined for the presence of white colonies, which signified successful uptake of the plasmid. Transformed colonies were picked and screened using a colony PCR approach. Briefly, eight 1 ml tubes were labelled per sample, and 50 µl Nuclease-free water added to each one. For each tube, a white colony was selected, labelled on the petri dish, then scraped off with a 10 µl pipette tip and pipetted into the tube, pipetting up and down to mix. From each tube, 10 µl was transferred into a labelled PCR tube, the tubes centrifuged briefly and then boiled at 95ºC for 6 minutes. The remainder of the solution was retained at four degrees for use in the next step, growing on and sequencing.

PCR was performed as follows, with 5 µl of each colony solution using the Avramenko / Mitchell primers and MyTaq Red polymerase. PCR conditions as previously with an annealing temperature of 55ºC and 40 cycles.

PCR products were visualised using gel electrophoresis on a 1% agarose gel run at 100 V as described previously, which showed that all the colonies had produced bands of the expected sizes.

4.3.12.3 Colony growth, plasmid extraction and sequencing

Colonies of interest were selected to be grown on for purification and sequencing. Colonies were grown overnight in round bottomed polypropylene tubes containing 7 ml of LB broth with 0.1% ampicillin. Remaining 40 µl colony solutions from colony PCR were added to the LB broth/ampicillin and the caps closed to halfway to allow gas exchange to take place. Cultures were incubated overnight at 37°C, shaking at 200 rpm.

Post overnight culture, if required, glycerol stocks were prepared. Stocks were prepared by mixing 100% glycerol with the grown-on colony liquid at a 1:1 ratio in a small, screw-topped tube and storing at -80°C.

Plasmids were purified using an Isolate II Plasmid mini-kit (www.bioline.com) according to the manufacturer's guidelines using 1.5 ml of each overnight culture. Each plasmid sample was eluted in 30 µl of nuclease free H_2O.

Plasmid DNA was again quantified using a NanoDrop 1000 spectrophotometer (www.thermofisher.com).

Plasmid DNA samples selected for sequencing were diluted to give 200 – 250 ng DNA in 5 µl total volume, prepared in 0.2 ml PCR tubes.

Plasmid DNA samples were prepared with either an M13 forward primer (Invitrogen, www.thermofisher.com) or an M13 reverse primer. Plasmid DNA was sequenced in house at Gogerddan campus, Aberystwyth University, using Sanger sequencing. The remaining plasmid DNA was stored at -20ºC until required.

Sequence data from Sanger sequencing were initially analysed in BioEdit (Hall, 1999) removing plasmid DNA overhangs. The trimmed sequence was then analysed using a BLASTn against the National Center for Biotechnology Information (NCBI) database without limiting for any species using the default parameters.

4.3.13 Barcoding samples to enable multiplex analysis on MinION

In order to sequence multiple samples through the MinION (Oxford Nanopore Technologies, Oxford, UK), it was necessary to label each sample with a barcode. This would permit all of the samples to be sequenced simultaneously, with a separate output from the basecalling for each sample that could be used to determine the species present.

A spreadsheet of barcodes was obtained from Dr Arwyn Edwards, Aberystwyth University. Each barcode comprised a leader sequence of 15 bases, which was conserved across all the barcodes, a unique 24 base barcode, and then the primer sequence itself. The barcodes would be used to provide a unique forward primer sequence for each sample, with the same reverse primer sequence used for all the samples. This would yield amplicons from each sample with a barcode "tag" enabling the samples to be discerned from each other when base-called using the MinION (https://nanoporetech.com/). Barcode sequences can be seen in Section 7.5 in the Appendix.

4.3.13.1 Barcode primer testing

Initially, three barcoded forward primers were purchased (www.sigmaaldrich.com), in order to determine whether it would be possible to produce the barcoded PCR products directly from the faecal DNA samples, or whether it would be necessary to perform a nested PCR with the second PCR cycle using barcoded primers. After the initial PCR confirmation, a further 26 barcodes were purchased giving a unique primer for each faecal DNA sample.

The 'Avramenko' primers were used for this section of work, because they had been successful in amplifying the ITS-2 region of the rDNA gene which would be sufficient to determine which species were present in the samples (Section 4.3.9). Two sets of PCR were performed simultaneously, using the protocol and PCR conditions from 4.3.9 albeit with only 35 cycles. The first set in each case used DNA from the faecal samples as the templates, and the second set used ITS-2 amplicons as the templates. Two negative controls were used for these experiments, one which had no template, and one which had a DNA template from a sample (Vinur) with a zero FEC.

4.3.13.2 First nested PCR

Having determined that the barcodes were best used as the second stage of a nested PCR, a template had to be produced from each sample, containing the ITS-2 amplicon. The protocol and PCR conditions from section 7.4 of the Appendix were used, with 35 cycles.

Repeat PCR was performed on those samples that did not show strong bands in the first attempt (5, 10, 13, 15, 16, 25 and 26) followed by a second repeat if required.

4.3.13.3 Second nested PCR: adding barcodes

In order to produce sufficient of the barcoded amplicons for MinION sequencing, three PCR runs for each sample were performed, using the individual barcoded primers as detailed in Section 7.5 of the Appendix. PCR conditions were as before, with repeat PCR runs as required.

4.3.14 MinION sequencing

Following faecal DNA sample barcoding, all samples were prepared for sequencing using the Oxford Nanopore Technologies Ltd MinION device; a small bench-top DNA sequencer (Figure 4-3).

Figure 4-3 Oxford Nanopore Technologies MinION sequencer, which provides benchtop sequencing in the laboratory of multiple samples simultaneously. The device is requires a connection to a PC and will be utilised for helminth sequencing for the first time.

Barcoded DNA samples were cleaned and purified followed by the addition of adapters to feed the strands of DNA through the pores in the MinION (https://nanoporetech.com/) to allow sequencing to be performed.

4.3.14.1 MinION sample preparation: clean-up

All the barcoded PCR products to be sequenced were defrosted at room temperature, and mixed together. A QiAgen gel clean-up kit (www.QiAgen.com) was used according to the manufacturer's instructions using 1.5 volumes of buffer QG (yellow) and omitting the isopropanol and initial incubation step.

Eluted DNA was measured on a Qubit (www.ThermoFisher.com) to ensure that each tube contained a DNA concentration of over 600 ng/µl. One of the three replicate samples was frozen at -20ºC as a back-up. The remaining two tubes were pooled for further clean-up.

4.3.14.2 MinION sample preparation: bead clean-up

A further clean-up step was performed using magnetic beads (https://www.beckmancoulter.com/). Two hundred µl of magnetic beads were added to the sample (this relatively large quantity of beads was used because the amplicons were short) and mixed by pipetting up and down. The tube was incubated at room temperature for eight minutes, agitating it from time to time to keep the contents mixed and then placed into a magnetic rack (Magnetic Separatron http://samandtomindustrys.science) so that the beads which had the DNA adhered to them, moved to the side of the tube against the magnet. The supernatant was removed using a pipette, and the beads washed with 80% ethanol. The tube was rotated on the rack so that the beads detached from the side of the tube, and then were re-attracted to the magnet. This step was repeated twice more, to give the DNA

coated beads a good wash. The ethanol was removed by pipetting, then the sample was briefly centrifuged and returned to the rack where any remaining ethanol was again removed. Thirty µl of 9 mM Tris pH 8 with 50 mM NaCl was added, and pipetted up and down to mix and incubated at room temperature for ten minutes. An aliquot of 1 µl was removed and checked on the Qubit Fluorometer to ensure that the DNA concentration had not reduced.

4.3.14.3 MinION sample preparation: Library preparation

The adapters which would feed the DNA strands through the nanopore in the MinION sequencing device were added using a LSK-108 library preparation kit (Oxford Nanopore Technologies Ltd) as per the manufacturer's instructions.

4.3.14.4 Preparation and loading of the MinION

Two labelled Lo-bind Eppendorfs were used for the library and for the MinION priming mixture. The priming mix contained 576 µl RBF (from the LSK-108 kit) well mixed before drawing off and 624 µl Nuclease-free water, and the library contained 35 µl RBF (again well mixed), 2.5 µl Nuclease-free water, 14 µl of the DNA preparation from step 4.3.14.3, making sure no beads were included and 25.5 µl of library loading beads (Oxford Nanopore Technologies Ltd).

The quality of the library was finally checked using Qubit and provided a reading of 22 ng/µl with a total volume of 75 µl giving 1.65 µg of DNA. A Flow Cell check was performed using the MinKNOW software (The MinION device software) which revealed 1,188 single pores functioning (469, 380, 253 and 8 in the four groups respectively). Each group contains 512 pores, and the Flow Cell check process examines each pore for blockages which would impede sequencing.

The flow cell was primed by loading 200 µl of the priming mix into the priming port (Figure 4-4) and leaving for ten minutes allowing the flow cell to prime. The DNA library was then loaded onto the flow cell by pipetting the entire 1.65 µg of library into the spot-on port (Figure 4-4). The loaded MinION was connected to a laptop and left overnight to sequence, controlled by MinKNOW software (https://nanoporetech.com/).

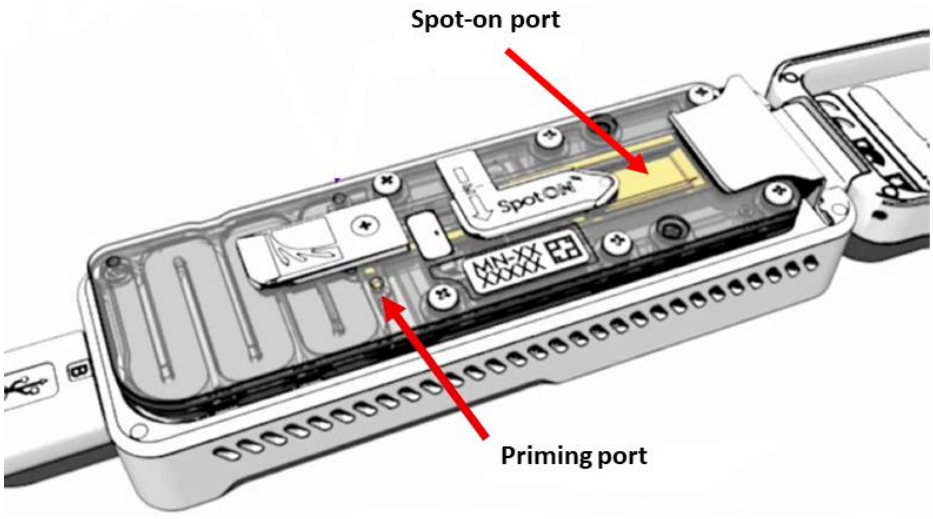

Figure 4-4 Oxford Nanoporetech MinION sequencer inside view, showing priming port and spot-on port used for loading DNA library samples; in this case nematode ITS-2 amplified products from equine DNA faecal extractions.

4.3.15 Data analysis

Having produced raw data reads from the MinION sequencing process, conversion into a list of the helminth species present in each sample, along with the proportions of each species DNA that was present in each sample, was required.

4.3.15.1 Data analysis: conversion of MinION output to DNA sequences

The raw output from the MinION produced FAST5 files. These data were run through the software program Albacore (Oxford Nanopore Technologies Ltd) which would provide a base-call; taking the electrical output from the MinION and converting it to and A, C, G or T, providing Albacore output files in FASTQ format. Albacore was set with a quality score cut off at greater than 7, separating reads into pass / fails.

The passing base-called reads from the were then analysed through PoreChop (https://github.com/rrwick/Porechop) which identifies and removes each barcode, and bins each FASTQ file into separate folders specific for each barcode. The min_split_read_size was set at 400, splitting reads with a central barcode and discarding fragments of less than 400 bp.

Following PoreChop analysis, data were analysed with CutAdapt (https://cutadapt.readthedocs.io/en/stable/) (Martin, 2011) which enables the primers to be removed and reads to be filtered through a quality control process. CutAdapt results in the data converted to FASTA format which was then analysed via BLASTn against the NCBI database to identify from which species each sequence was derived. In total, ten different parameter runs were assessed on CutAdapt.

1. Remove forward and reverse primers (if found) and trim to 250 bases.
2. Remove forward and reverse primers (if found) and discard if length < 100 bases.
3. As run 2 but discard if both primers not found.
4. As run 3 but discard if the quality scores is < 15.

5. Remove forward and reverse primers (if found) and discard if length < 220 bases.
6. Remove forward and reverse primers (if found) and discard if length < 500 bases. (To quality control the data set to see if every sequence was discarded).
7. Remove forward and reverse primers (if found), trim sequence to 250 bases and discard if < 220 bases.
8. Remove forward and reverse primers (if found), trim sequence to 400 bases and discard if < 220 bases. (Given the predicted amplicon was predicted to be approximately 400 bp long).
9. As run 8 but discard if < 350 bases.
10. Remove forward and reverse primers (if found) and discard if > 500 or < 350 bases.

NB In runs where trimming occurred, bases were trimmed equally from the 5' and the 3' ends, to remove low quality parts of the read.

4.3.15.2 Data analysis: BLASTn searches to identify species

Outputs from CutAdapt were compared against the NCBI database (https://www.ncbi.nlm.nih.gov/) using a BLASTn analysis. Initially, an output was produced that reported the top sequence identity, but it became apparent that this often produced good matches but over only a short sequence length, which consequently had a high e-value (a high potential for the match to be coincidental). Owing to this, it was decided to report only sequences with a certain percentage identical match, and three sensitivities were chosen – 97%, 95% and 90%. From these results, any with an alignment length of less than 35 bases (10% of the

sequence length as per Avramenko *et al.* (2015)) were discarded, as were any with e-values which were greater than 0.001. The remaining sequences were sorted and summarised by species.

Using the 90% sensitivity, it became apparent that most of the expected matches (i.e. cyathostomin species) had good long alignment lengths of 300 – 350 bases. Therefore, discarding alignment lengths under 100 bases to further increase accuracy was trialled on two barcode runs (barcode 3 and barcode 8).

4.3.15.3 Data analysis: sequence proportion comparison

The species identified from each sample were quantified, and the proportions of each calculated. Given that the total number of sequences produced in each sample was more a factor of the success of the DNA extraction all calculations were performed on relative proportions of each nematode species. In total, six principal species were identified along with those species which constituted less than 5% of the total sequences in any sample which were collected together and termed "Minor species" for the rest of the data analysis.

4.3.15.4 Data analysis: effect of treatment on each principal species

A fourth root transformation and Bray Curtis similarity analysis was performed on the sequence proportions, and visualised using an MDS plot. Scatter plots were produced for each species, plotting post-treatment sequence proportions from each horse against pre-treatment sequence proportion. Trend lines were added, to go through the origin, illustrating the percentage increase or decrease following treatment, and R^2 values added to indicate how strongly this relationship was explained by the data. P values were produced using the "Regression" feature of the

data analysis plug-in for Microsoft Excel, to demonstrate whether any relationship between pre- and post-treatment sequence proportion of a species was significant.

The analysis was repeated plotting change in sequence proportion against anthelmintic treatment efficacy (the proportion that the FEC reduced in each sample two weeks after treatment). P values were produced in the same manner. These calculations were repeated twice, once using the actual change in sequence proportion, and once using the percentage change in sequence proportion. This was done in order to take into account the differing levels of each species – for example, a change in sequence proportion from 5% to 10% would be more relevant than a change from 60% to 65% even though the actual change would still be 5. This avoided over-stating the importance of those species that were not highly represented.

Initially, it had been hoped that a comparison could be made between the species found at the three different locations. However, due to the low levels of parasitic infections identified, there were insufficient samples tested at two of the locations for the results to be meaningful. Contingency tables were produced to investigate whether the pre- or post-treatment samples were significantly different from each other in the three locations, and also whether they were significantly different from each other across all the samples. The principal species were used as the row variables, with the pre- or post-treatment sequence proportions as column variables. Pie charts were also produced to demonstrate the proportions of sequences found in each of the locations.

To assess if there was a relationship between FEC and the diversity of species identified within each sample, the Shannon index of species diversity was calculated

using the actual number of sequences observed in each sample. Actual sequence numbers were used in this instance in order not to filter out those species where the sequences made up less than 1% of the total. When comparing Shannon indices, the size of the population is not relevant, so in this case sequence numbers rather than proportions could be used with no detriment. The Shannon index for each sample was calculated using the formula:

$$H' = -\sum_{i=1}^{R} p_i \ln p_i$$

Where, in the equation above, p_i is the proportion of sequences in the *i*th species. P values were calculated using the Regression function of the data analysis plug-in for Microsoft Excel, to demonstrate if there was a significant correlation between FEC and Shannon index for either pre- or post-anthelmintic treatment samples, or between the Shannon index of the pre- and the post-treatment samples.

4.4 RESULTS

4.4.1 Resistance testing

In order to assess anthelmintic effects on the equine nemabiome it was important to determine if there was any nematode resistance present to the anthelmintic compounds tested and therefore faecal samples containing nematode eggs from resistant populations (from the post-treatment samples) and mixed populations (from the pre-treatment samples) were collected for genetic sequencing.

Overall, the mean reduction in strongyle nematode FEC fourteen days after treatment with Ivermectin was 100% ± 1%. Infection levels of those horses that were treated ranged from 221 epg to 1987 epg (Mean overall of treated horses 587 epg, yard one mean 607 epg, and yard two mean 560 epg). When analysed via location, thirteen of the samples on yard 1 had pre-treatment FECs of below 200 epg, and therefore were not treated. Two of the horses from the study were moved before post-treatment samples could be collected. The mean reduction post-treatment of the remaining ten horses was 100%. It was interesting to note the presence of *Parascaris equorum* nematode eggs in one of the post-treatment samples. In yard two, sixteen of the samples had pre-treatment FECs of below 200 epg, and of the nine treated horses, the mean reduction in FEC was 99%.

When treating with Fenbendazole, across all yards, fifty two of the equine faecal samples tested had pre-treatment FECs of less than 200 epg, and thus were excluded from further testing, due to being under the selected treatment threshold (Coles, 2009) . Of the fifteen horses that were treated, no samples provided evidence that Fenbendazole was effective at removing the worm burden. Post-treatment FEC

reductions ranged from 0% to 74% with a mean reduction of 38% ± 23% (Figure 4-5). Of interest, was the identification of one horse sample (Jigsaw) that had a pre-treatment *P. equorum* FEC of 327 epg that was reduced to zero post-treatment.

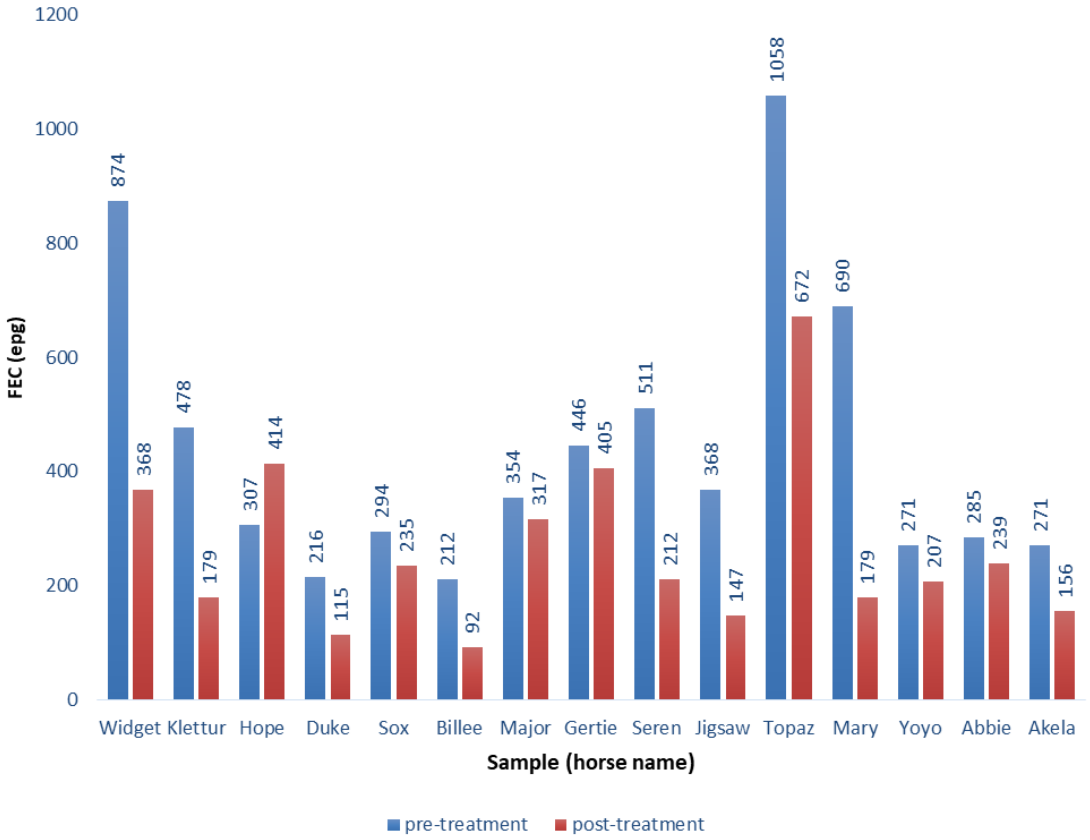

Figure 4-5 Reduction in equine strongyle nematode FEC 14 days post treatment with Fenbendazole. Blue bars are pre-treatment FECs and red bars represent post-treatment FECs. The numbers given above each bar provide the actual epg for ease of comparison.

All pre- and post-treatment samples were retained for future DNA nemabiome analysis.

4.4.2 Molecular genetics

Genetic sequencing of DNA extracted and amplified from equine faecal samples, collected both pre- and post-treatment, was attempted to determine the nematode species composition constituting the equine nemabiome.

4.4.3 Primer selection: rDNA

In order to determine which species were present, a portion of the ribosomal gene would be amplified using PCR. This gene is highly conserved among nematodes, and as the MinION is a powerful sequencer, amplification of the largest possible segment of this gene was hoped for.

Primers selected from the literature for rDNA sequencing are provided in Table 4-3.

Table 4-3 Small sub-unit rDNA primers selected from the literature for nemabiome amplification assessment.

Paper	Forward primer	Reverse primer	Product size	Species	Annealing temp
Marek *et al.* (2010)	5'-TTGATTAGGTCCCTGCCCTTT-3'	5'-TTTCACTCGCCGTTACTAAGG-3'	all 18s rDNA gene	Plant nematodes	60º
Powers *et al*. (1997)	5'-TTGATTACGTCCCTGCCCTTT-3'	5'-GTAGGTGAACCTGCAGATGGAT-3'	ITS1 5.8s ITS2 155bp plus ITS1 and 2 total 425bp	Wide range of nematodes	57º
Peachey *et al.* (2017)	5′ GATTGATTCTGTCAGCGCTATA 3′	5′ TAATGAGCCGTTCGCAGT 3'	18s 99bp	Various, including 6 cyathostomin species	53º
Floyd *et al.* (2005)	5'-CGCGAATRGCTCATTACAACAGC-3'	5'-GGGCGGTATCTGATCGCC-3'	Fragment of 18s approx. 900 bp	All available nematode species (excludes other contaminants)	54º
Avramenko *et al*.(2015) also Mitchell *et al.* (2019) and Andersen *et al.* (2013)	5 '-ACGTCTGGTTCAGGGTTGTT-3'	5 '-TTAGTTTCTTTTCCTCCGCT-3'	ITS2 region 250bp	Cattle nematodes (Mitchell and Andersen used it on horses)	55º

4.4.4 DNA Extraction: QiAgen kit

The first priority in the DNA extraction process was to assess extraction methods, and the first method tested was a commercial kit.

Using the QiAmp DNA stool kit, but with the addition of a bead disruption step, the final amount of DNA produced from a representative test sample was measured on a NanoDrop spectrophotometer (https://www.thermofisher.com/uk/en/home.html), was 7.8 ng/µl with 260/280 and 260/230 ratios of 1.74 and 0.71 respectively. The DNA was examined on a 1% Agarose gel run at 100 V, to confirm quality (Figure 4-6 a) and demonstrated high molecular weight DNA.

Using the QiAmp DNA stool kit as per manufacturer's instructions, with no bead disruption step, the final amount of DNA produced from the test sample when analysed on a NanoDrop spectrophotometer was 31.0 ng/µl with 260/280 and 260/230 ratios of 1.71 and 1.52 respectively. The DNA was visualised on a 1% Agarose gel run at 100 V, to confirm quality (Figure 4-6b). It can be seen that this DNA produced more of a band at the top than that produced with the bead disruption step (Figure 4-6a), showing a greater proportion of high molecular weight DNA.

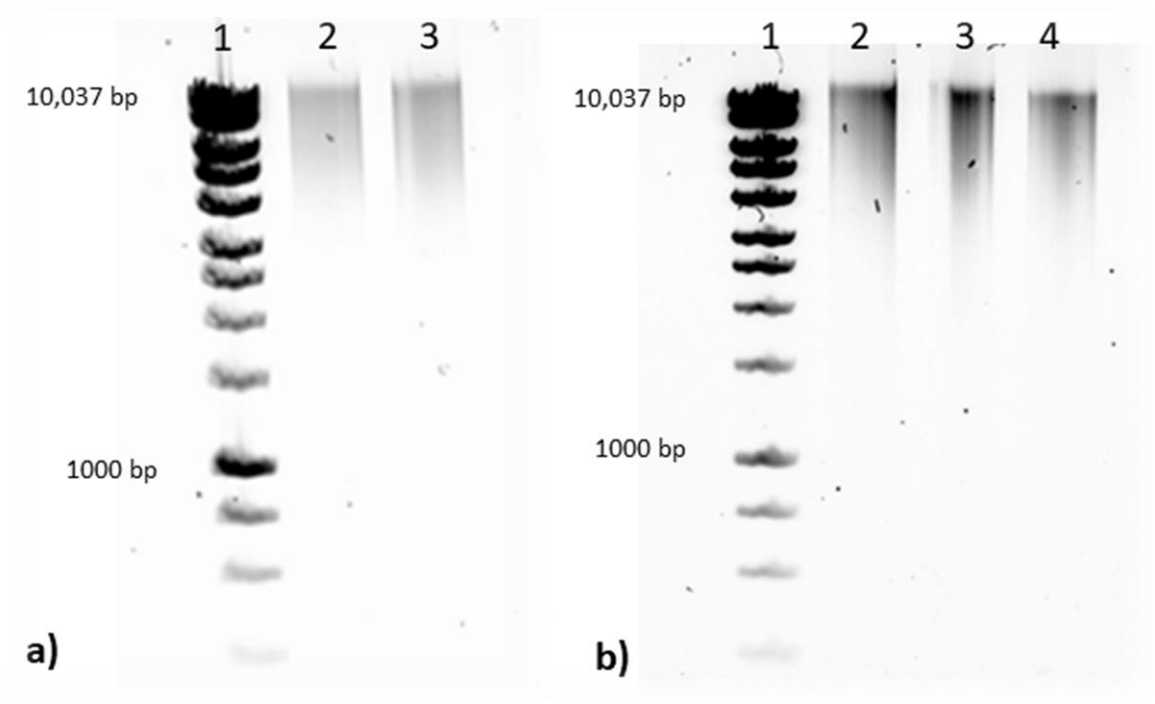

Figure 4-6 Equine faecal DNA extraction using QiAgen kit analysed on a 1% agarose gel. a) With bead disruption step. Lane 1shows 1kb ladder (Bioline) DNA can be observed at the top of lanes 2 and 3. b) Without bead disruption step. Lane 1shows 1kb ladder (Bioline) DNA can be observed at the top of lanes 2, 3 and 4.

4.4.4.1 DNA Extraction: QiAgen kit freeze drying

In order to discover whether a greater amount of starting material could be used for the DNA extraction, it was decided to try freeze drying some samples. Klettur pre-treatment sample was chosen for this experiment which had a FEC of 478 epg. The quantity of DNA extracted is presented in Table 4-4 below, which shows that using freeze dried faecal material and along with a bead disruption step produced a greater amount of DNA which was of greater quality than the other methods.

Table 4-4 Quantity of DNA extracted from freeze-dried and frozen equine faecal samples as measured on a NanoDrop spectrophotometer, extracted with the QiAgen QiAmp stool kit either with or without a bead disruption step. Both 260/280 and 260/230 ratios are given as an indicator of DNA purity.

Method	DNA ng/µl	260/280	260/230
1. Frozen	6.9	1.21	0.80
2. Freeze dried	5.7	1.41	0.85
3. Frozen with beads	5.9	1.70	0.33
4. Freeze dried with beads	10.1	1.76	0.68

Two replicates of DNA extraction were performed using the QiAgen stool kit and a bead-beating step using a Tissue Lyser (QiAgen, Hilden, Germany). This yielded DNA of 5.8 ng/µ (260/280 ratio 1.58 and 260/230 ratio 0.57) and 11.9 ng/µ (260/280 ratio 1.83 and 260/230 ratio 0.73) when measured on the NanoDrop.

A summary of the NanoDrop readings from all the DNA samples which were extracted using the QiAmp DNA Stool kit with additional modifications is provided in Table 4-5. Only one of the DNA extraction samples, the first extraction on Gertie pre, gave a reasonable quantity and quality of DNA using this extraction method.

Table 4-5 Summary of quantities of DNA extracted from faecal samples using QiAgen QiAmp DNA Stool kit and with modified protocols. All DNA quantities and ratios were measured on a NanoDrop spectrophotometer.

Sample and protocol	ng/ul	260/280	260/230
Without beads			
Major pre	7.8	1.74	0.71
Gertie pre	31.0	1.71	1.52
Klettur pre	6.9	1.21	0.80
Widget pre	7.9	1.41	0.38
Widget post	3.9	2.10	0.50
Klettur pre	4.0	1.49	0.30
Klettur post	4.6	1.62	0.31
Seren pre	2.7	1.28	-0.55
Seren post	4.0	1.35	-3.18
Major pre	2.8	1.30	-0.94
Major post	3.5	1.51	5.09
Gertie pre	8.1	1.50	0.69
Gertie post	7.6	2.57	0.46
Akela pre	12.0	2.03	0.62
Akela post	4.3	1.97	0.80
Freeze dried without beads			
Klettur pre	5.7	1.41	0.85
With beads			
Klettur pre	5.9	1.70	0.33
Freeze dried with beads			
Klettur pre	10.1	1.76	0.68
Tissue Lyser			
Topaz pre	5.8	1.58	0.57
Topaz post	11.9	1.83	0.73

4.4.5 DNA Extraction: CTAB method

Given the low quantity of extracted DNA using the commercial QiAgen extraction kit, an alternative method was assessed. The chosen method followed was that used by Mitchell *et al*. (2019) which had been adapted from Yu and Morrison (2004), Dellaporta *et al.* (1983) and William *et al.* (2012).

4.4.5.1 DNA Extraction: CTAB method, comparison between freeze-dried and frozen sample

In order to assess the use of freeze dried and fresh frozen faeces a single replicate of a CTAB DNA extraction was performed on an equal volume of a freeze-dried and a frozen aliquot of the same faecal sample. The freeze-dried sample yielded 481 ng/µl DNA (260/280 ratio of 1.44 and 260/230 ratio of 1.20) whereas the frozen sample yielded almost 6-fold more concentrated DNA at 2,802 ng/µl (260/280 ratio of 1.86 and 260/230 ratio of 1.62) when measured on a NanoDrop spectrophotometer.

Given the greater quantity of DNA extracted via the CTAB method, DNA was then extracted from all samples using an aliquot of approximately 200 mg from each of the frozen faecal samples. The DNA was quantified using the NanoDrop as before, providing DNA levels of greater than 315.2 ng/ µl (Table 4-6).

Table 4-6 Summary of quantity of DNA extracted from equine faecal samples using CTAB method, measured on a NanoDrop spectrophotometer and Qubit Fluorometer. Mean NanoDrop 1052 ng/µl ±604 Mean Qubit 113 ng/µl ±59

Sample (horse name)	NanoDrop ng/ul	260/280	260/230	Qubit ng/µl
Widget pre	1211.8	2.05	2.18	176
Widget post	2802.0	1.86	1.62	195
Klettur pre	970.8	1.90	1.61	125
Klettur post	647.9	1.82	1.38	76.2
Seren pre	394.6	1.97	2.01	70.8
Seren post	945.1	1.94	1.69	113
Jigsaw pre	629.3	1.91	1.55	89
Jigsaw post	921.3	2.04	2.13	112
Major pre	315.2	1.91	1.70	32
Major post	447.5	1.90	1.77	69.2
Gertie pre	540.4	1.87	1.75	102
Gertie post	814.1	1.97	1.73	102
Hope pre	1106.1	1.80	1.39	106
Hope post	1549.4	1.89	1.63	150
Billee pre	1662.6	2.02	2.07	152
Billee post	484.7	1.83	1.59	54.4
Duke pre	2303.6	2.00	2.08	290
Duke post	1692.9	2.04	1.99	154
Sox pre	1141.9	1.98	1.84	110
Sox post	1723.2	1.83	1.56	216
Topaz pre	590.1	1.90	1.84	47.2
Topaz post	537.9	1.95	1.95	65.6
Mary pre	1070.8	1.93	1.67	118
Mary post	1185.9	2.07	2.37	121
Yoyo pre	906.7	1.69	1.14	36.4
Yoyo post	754.1	1.78	1.27	67.8

DNA was manually inspected using gel electrophoresis on a 1% Agarose gel run at 100 V, to visualise the extracted DNA. (Figure 4-7), showing good quality DNA.

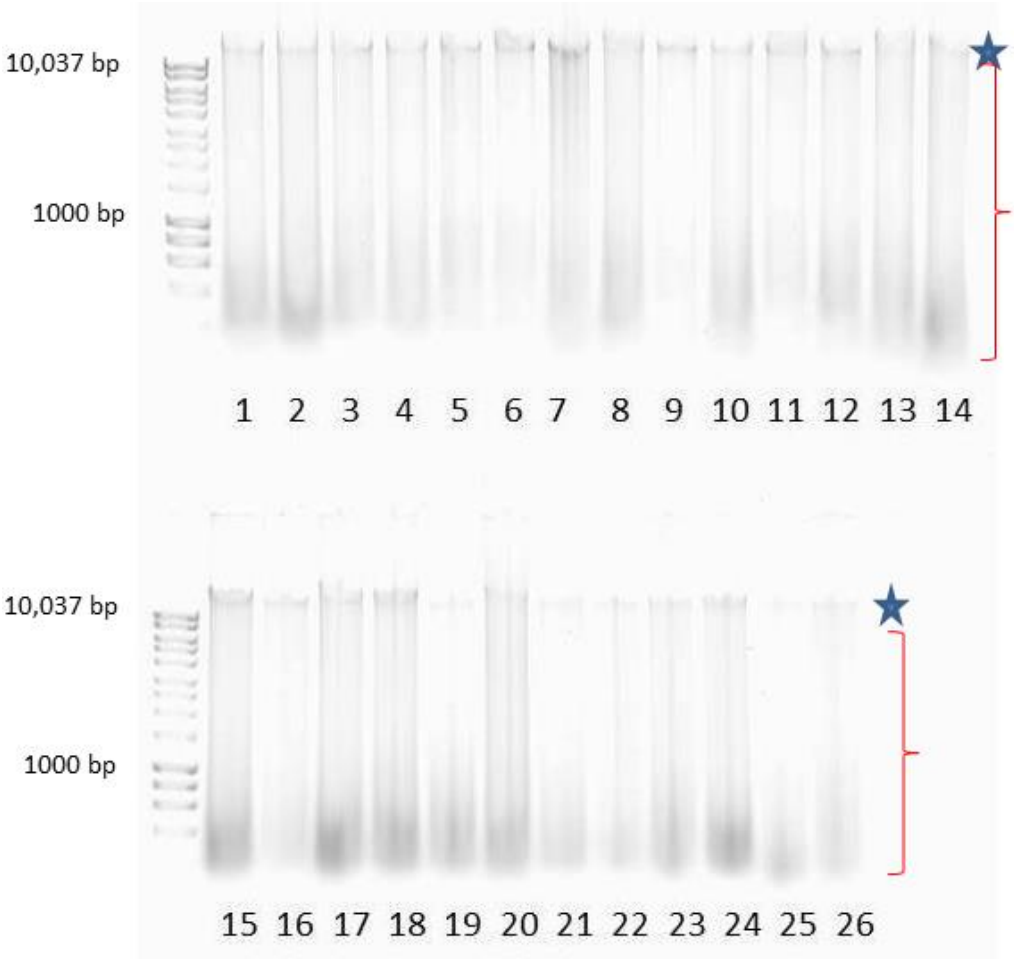

Figure 4-7 Assessing the quality of DNA extracted from equine faecal samples using the CTAB method. 1: Widget pre, 2:. Widget post, 3: Klettur pre, 4: Klettur post, 5:. Seren pre, 6:. Seren post, 7: Jigsaw pre, 8: Jigsaw post, 9: Major pre, 10: Major post, 11: Gertie pre, 12: Gertie post, 13: Hope pre, 14: Hope post, 15: Billee pre, 16: Billee post, 17: Duke pre, 18: Duke post, 19: Sox pre, 20: Sox post, 21: Topaz pre, 22: Topaz post, 23: Mary pre, 24: Mary post, 25: Yoyo pre, 26: Yoyo post. Pre denotes before anthelmintic treatment and post signifies samples 14 days after anthelmintic treatment. Ladder (1 kb Hyperladder, Bioline) on the left hand lanes. Stars indicate DNA bands, brackets indicate RNA.

It can be observed that both DNA (indicated by stars) and RNA (indicated by brackets) are present in DNA extraction samples (Figure 4-7). No RNAse treatment was used, as RNA would not interfere with the DNA sequencing. Given the

NanoDrop spectrophotometer quantification measures both DNA and RNA, all DNA extractions were also quantified using a Qubit 2.0 Fluorometer (www.thermofisher.com) which quantifies only DNA (Figure 4-6).

As expected, the Qubit readings for the DNA concentrations were lower than those obtained from the NanoDrop spectrophotometer which also included RNA.

4.4.6 DNA Extraction from FECPAK^{G2} Cassette preparations

In order to link nematode FECs, via the FECPAK^{G2} to molecular species determination, this section of work aimed to determine if the samples prepared for imaging using the cassette and Micro-I could be used for downstream DNA analysis. This would potentially enable an efficient workflow to detect and quantify a parasitic infection, and then also determine the species present.

4.4.6.1 DNA extraction from FECPAK^{G2} Cassette preparations

Firstly, the QiAgen QiAmp stool kit was used to extract DNA from the FECPAK^{G2} cassette preparations. Initial NanoDrop results from the first attempt was based upon a sample that was centrifuged and washed prior to the extraction and provided extremely low DNA levels of 0.8 ng/µl (260/280 ratio of 1.81 and 260/230 ratio of -0.43) likely indicating that the DNA extraction may not have been successful. Given the DNeasy protocol suggests washing the sample in phosphate buffered saline it seemed likely that saline would not inhibit the extraction. Therefore, DNA extraction was repeated without washing the eggs from the saline to avoid potential egg loss during these wash steps.

Following extraction of the FECPAK^{G2} cassette prepared sample which was not washed a concentration of DNA at 2 ng/µl (260/280 ratio of 6.34 and 260/230 of 0.32) was achieved. As these two samples had been stored at -20ºC it was decided to repeat the extraction using a fresh faecal sample prepared using the FECPAK^{G2} method, and this sample was also prepared using water instead of saline, to determine if the saline was interfering with the DNA extraction process. A centrifugation step was also added to the final replicate.

NanoDrop results from the samples prepared from a fresh faecal preparation were as follows:

Sample 1 (saline) 3.5 ng/µl 260/280 1.26 260/230 0.57

Sample 2 (water) 2.3 ng/µl 260/280 1.01 260/230 0.86

Sample 3 (centrifuged) 4.2 ng/µl 260/280 1.26 260/230 0.58

The DNA extraction was also attempted from the FECPAK^{G2} cassette preparations using the CTAB method. Despite the previous success of the CTAB method the NanoDrop results for the on the FECPAK^{G2} Cassette samples was again significantly low. Of the two replicates assessed, only one gave a positive DNA concentration at 0.3 ng/µl (Klettur: 260/280 ratio of 0.39 and 260/230 ratio of 0.14). The second replicate produced a concentration at -0.4 ng/µl (Widget: 260/280 ratio of 0.92 and 260/230 ratio of -0.29).

4.4.7 PCR optimisation: rDNA

Following successful DNA extraction either from the Qiagen kit or the CTAB method, PCR primers and PCR conditions were assessed for amplifying a segment

of the small sub-unit ribosomal gene, to enable the species of helminths contained in the samples to be determined.

4.4.7.1 PCR optimisation: rDNA initial selection

Initially, the primers were tested on a *B. glabrata* DNA sample as a potential positive control in addition to DNA extracted from an equine faecal sample which had yielded the greatest concentration of DNA when extracted using the QiAgen kit.

Following PCR amplification, all products were analysed on a 1% w/v Agarose gel (Figure 4-8a). Of significant interest was a weak product that was observed using the 'Peachey' primers and the equine DNA extraction, although this was larger than the anticipated 99 bp size at approximately 200 bp. In addition, strong banding at the expected size for both the 'Marek' and 'Floyd' primer sets were also observed, but only to the positive control (Figure 4-8b).

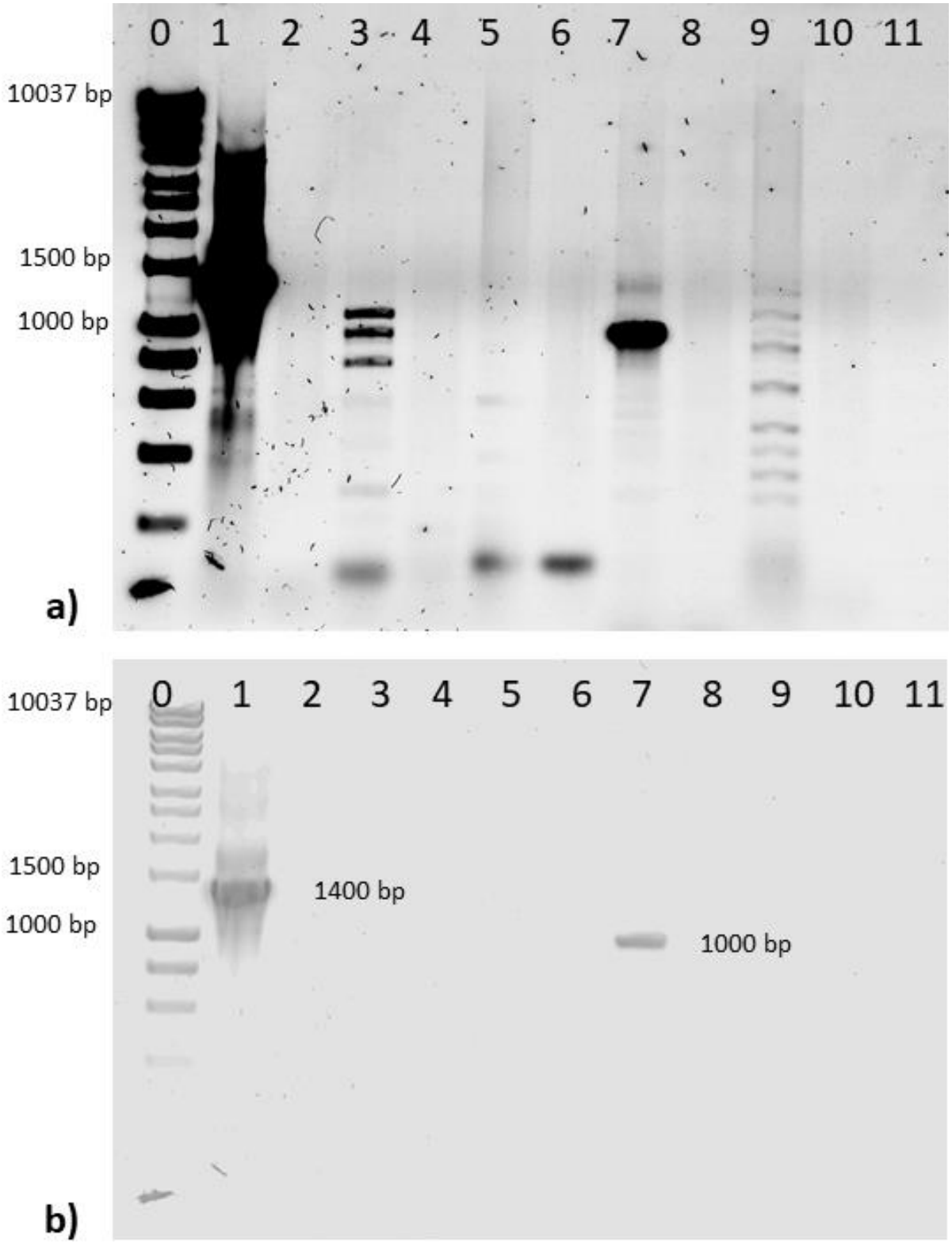

Figure 4-8 PCR primer optimisation for equine faecal analysis a) PCR products from initial rDNA primer comparison, with the contrast adjusted to visualise weak bands and b) with the original contrast settings. (Lanes: 0: 1 kb ladder, 1: Marek control, 2: Marek sample, 3: Powers control, 4: Powers sample, 5: Peachey control, 6: Peachey sample, 7: Floyd control, 8: Floyd sample, 9: Avramenko control, 10: Avramenko sample, 11: Negative control)

A second replicate was also performed using the DNA extracted from a different equine faecal sample with 11.4 ng/µl. Only the ‘Floyd’, ‘Marek’ and ‘Peachey’ primers were assessed in this replicate given they had produced PCR products on the previous attempt.

As before PCR products were imaged using gel electrophoresis. However, no products were visible other than for the positive control for which a band of approximately 1,000 bp was observed (Figure 4-9a & Figure 4-9b).

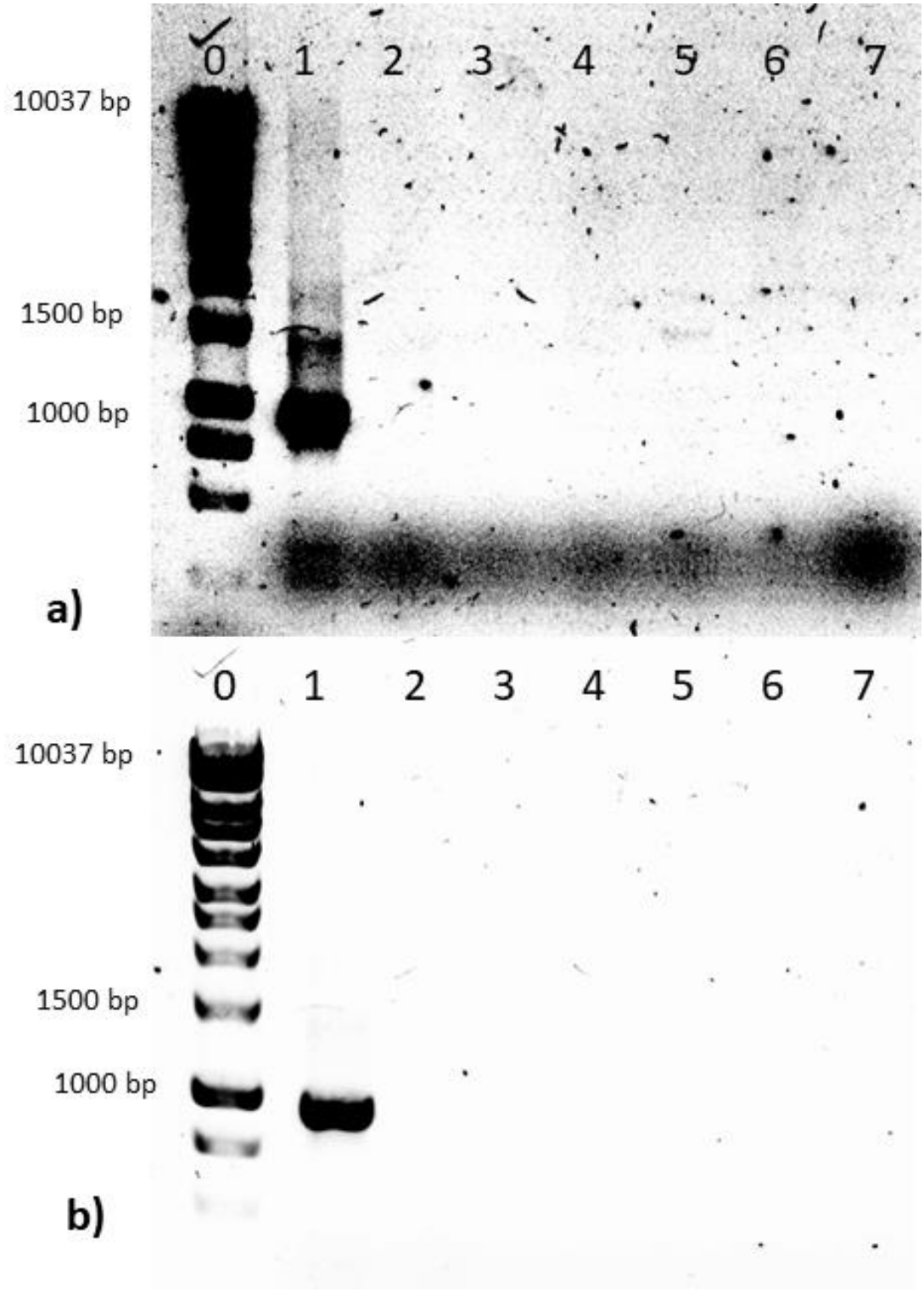

Figure 4-9a) PCR primer optimisation for equine faecal analysis a) PCR products from second rDNA primer comparison, with the contrast adjusted to visualise weak bands and b) with the original contrast settings. (Lanes: 0: 1 kb ladder, 1: Floyd control, 2: Floyd sample, 3: Floyd negative control, 4: Marek sample, 5: Marek negative control, 6: Peachey sample, 7: Peachey negative control).

4.4.7.2 PCR optimisation: rDNA using helminth DNA

Given the success of certain primers amplifying snail DNA, they were optimised for nematodes using Ascarid DNA. As previously the 'Floyd', 'Marek' and 'Peachey' primers were assessed. Following PCR product analysis on a 1% Agarose gel, only a product was amplified for the snail DNA control at approximately 1,000 bp, and not for the Ascarid DNA with any primer set (Figure 4-10).

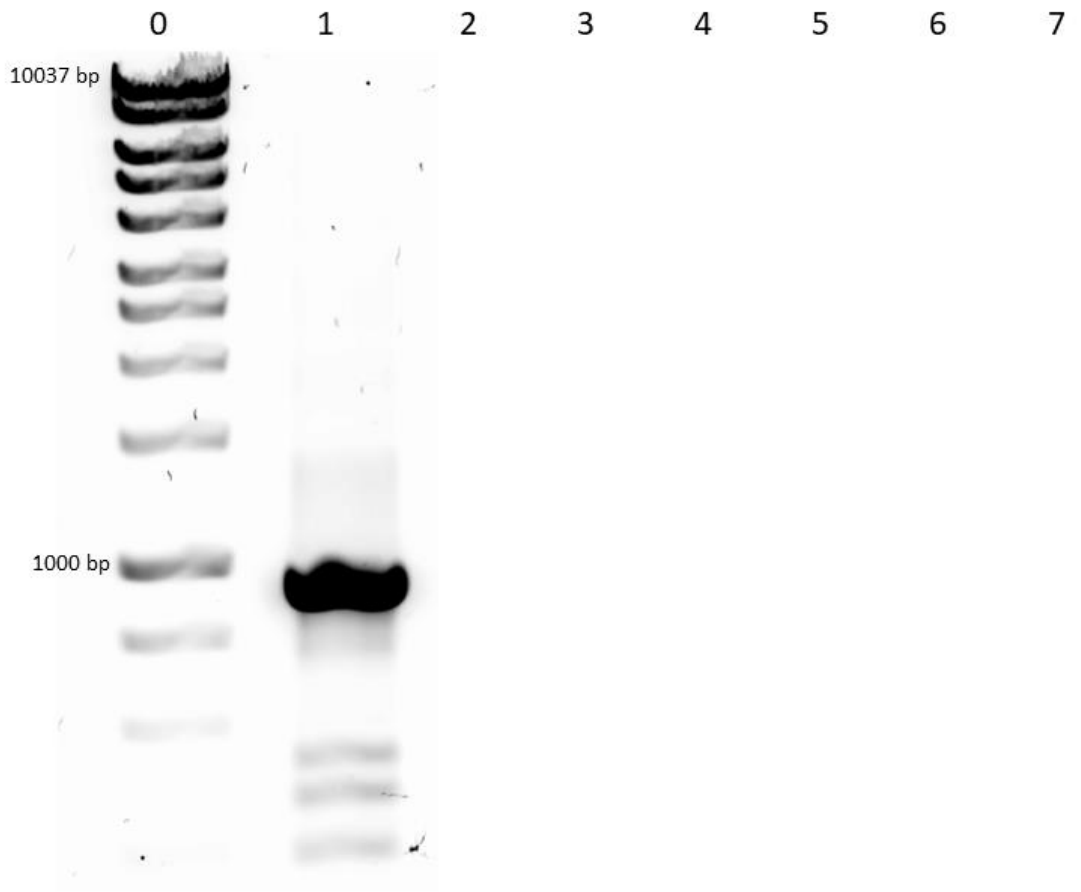

Figure 4-10 PCR primer optimisation for equine faecal analysis using Ascarid DNA. (Lanes: 0: 1 kb ladder, 1: Floyd control, 2: Floyd Ascarid, 3: Floyd negative control, 4: Marek Ascarid negative control, 5: Marek negative control, 6: Peachey Ascarid, 7: Peachey negative control).

As the quality of the Ascarid DNA was unknown, a sample of DNA from somules of the parasitic flatworm *Schistosoma mansoni* was used to test the primers to see if a product could be obtained from alternative helminth DNA.

The PCR products were imaged on a 1% w/v Agarose gel run at 100 V with the 'Marek' and 'Floyd' primer sets providing strong products with the schistosomule DNA. However, the 'Marek' product was smaller than when amplified on snail DNA (Figure 4-11). There were also additional products observed following amplification

with the 'Peachey' primer set, although these were all larger than the 99 bp expected product size and only weak bands.

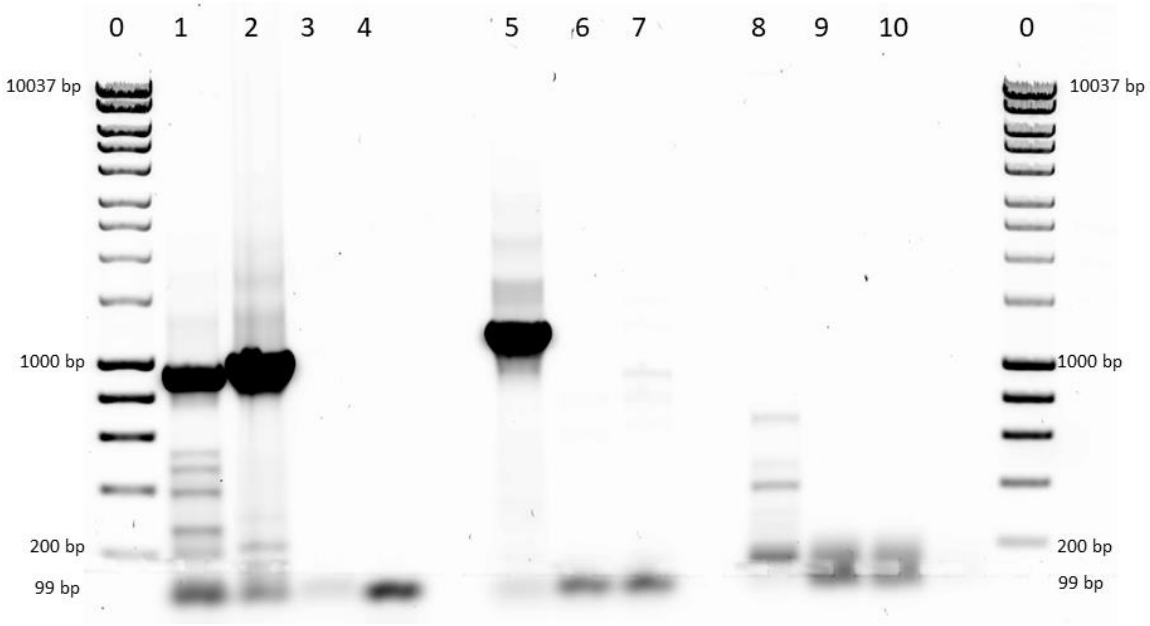

Figure 4-11 PCR products assessing rDNA primers used with schistosomule and *Ascaris* DNA (Lanes: 0: 1 kb Hyperladder, 1: Floyd control, 2: Floyd schistosomule, 3: Floyd Ascaris, 4: Floyd negative control, 5: Marek schistosomule, 6: Marek Ascaris, 7: Marek negative control, 8: Peachey schistosomule, 9: Peachey Ascaris, 10: Peachey negative control).

With no amplification observed for the Ascarid DNA, fresh DNA was extracted from a female Ascarid worm and used for all further experiments. As there was a doubt on the quality of the worm itself, a commercially produced chemically preserved sample, DNA was also extracted from *Panagrellus redivivus*, a free-living nematode, as a further attempt to see if a nematode control could be acquired.

All three primer sets produced bands with both the Schistosomule DNA and the *P. redivivus* DNA but once again the Ascarid DNA failed to produce a band with any of the primer sets tested (Figure 4-12). The 'Floyd' primers produced the strongest

products with the nematode, *P. redivivus* DNA and thus chosen as the primers to use for further work.

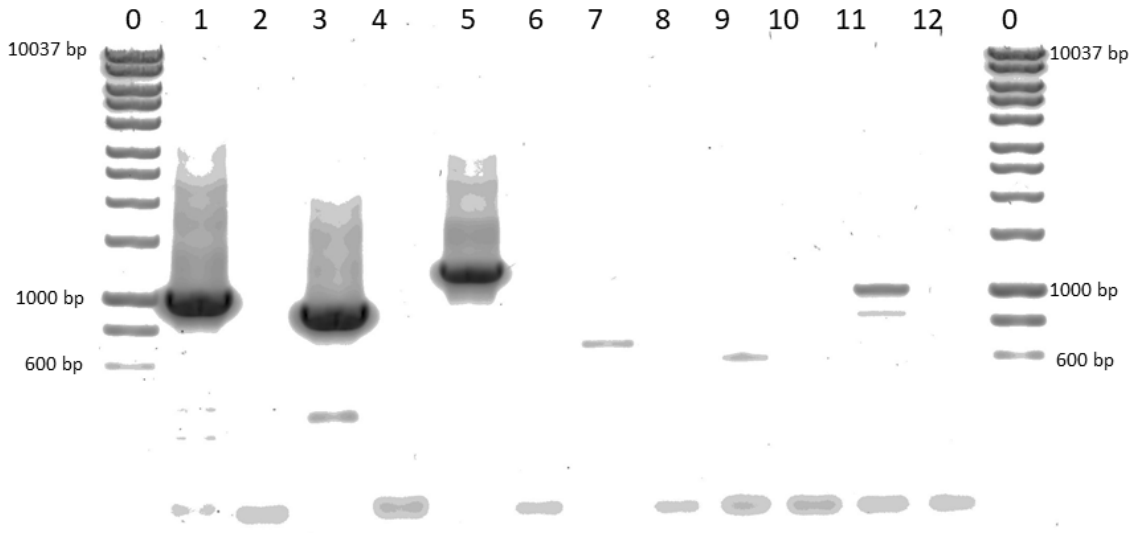

Figure 4-12 PCR products assessing rDNA primers used with schistosomule and *Ascaris* and *P. redivivus* DNA (Lanes: 0: 1 kb Hyperladder, 1: Floyd schistosomule, 2: Floyd negative control, 3: Floyd *P. redivivus* 4: Floyd Ascarid, 5: Marek schistosomule, 6: Marek negative control, 7: Marek *Panagrellus*, 8: Marek Ascarid, 9: Powers schistosomule, 10: Powers negative control, 11: Powers *P. redivivus*, 12: Powers Ascarid)

4.4.8 PCR of DNA extracted using QiAgen kit

Following selection of suitable PCR primers the aim of this work was to amplify a region of parasite DNA from the DNA extractions on faecal samples, using the extraction conditions and the primer set that were considered to perform the best. The 'Floyd' primers had been selected as the most promising ones to use, and 12 faecal samples were processed using the optimal method of extraction using the QiAgen kit (frozen samples, without a bead disruption step), then the samples were tested using PCR.

The PCR products were imaged using gel electrophoresis (Figure 4-13) and demonstrated a variety of amplified products all of which were of different bp sizes when compared to the positive control DNA.

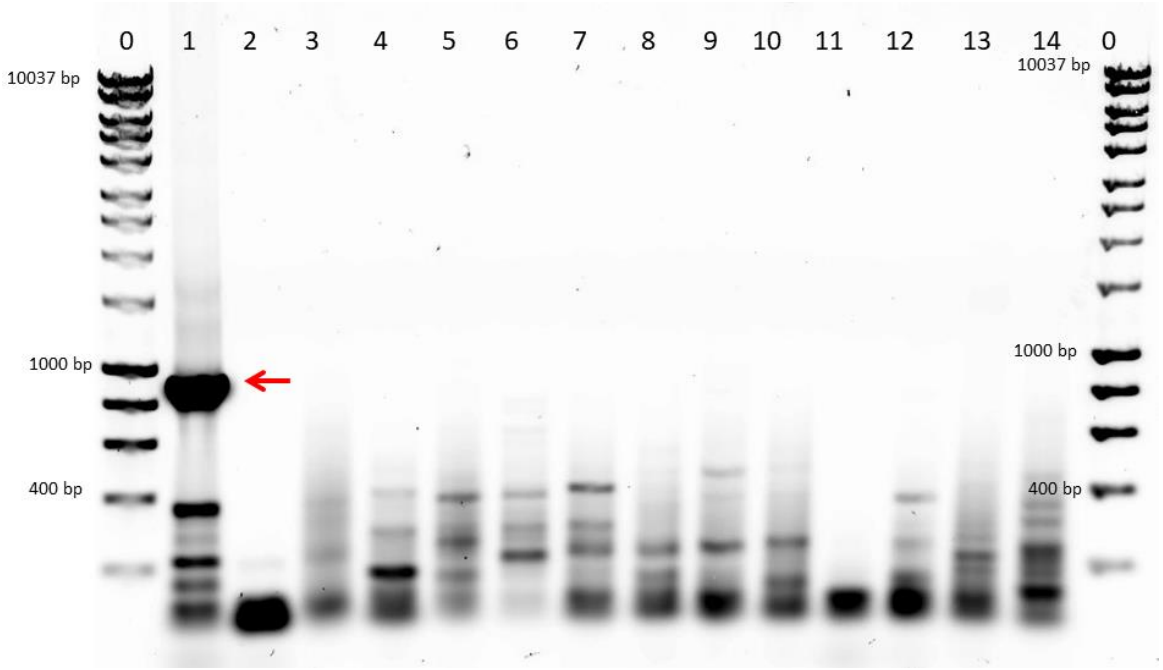

Figure 4-13 PCR products assessed using 'Floyd'rDNA primers and DNA extracted using QiAgen kit (Lanes: 0: 1 kb Hyperladder 1: Positive control (*P. redivivus*), 2: Negative control, 3: Widget pre, 4: Widget post, 5: Klettur pre, 6: Klettur post, 7: Seren pre, 8: Seren post, 9: Major pre, 10: Major post, 11: Gertie pre, 12: Gertie post, 13: Akela pre, 14: Akela post). Product of expected size produced from positive control, highlighted by red arrow.

4.4.9 PCR of DNA extracted using CTAB method

Following the limited success of PCR amplification with the 'Floyd' primer set on DNA extracted with the QiAgen kit, amplification was assessed using the CTAB extracted samples. In total, ten faecal samples were processed using the CTAB method of extraction and analysed using PCR. The amplification was unsuccessful, as was a second replicate with the template DNA reduced to 20 ng (data not shown).

As the ‘Floyd’ primer set had been unsuccessful at amplifying the DNA extracted from the faecal samples, it was decided to try one of the other primer sets. Using 20 ng of template DNA in each reaction and using the ‘Avramenko’ primer set that had successfully been used to speciate cyathostomins in Mitchell *et al*. (2019) a good PCR product was observed for every sample of DNA extracted from a faecal sample using the CTAB method (Figure 4-14). The products produced for the samples were at approximately 400 bp, whereas for the control it is 200 bp. The expected size of the product when amplifying cyathostomin DNA was 400 bp, as seen in Mitchell *et al*.(2019). It was interesting to note that two bands were observed in samples 7 and 8 (Major pre and Major post).

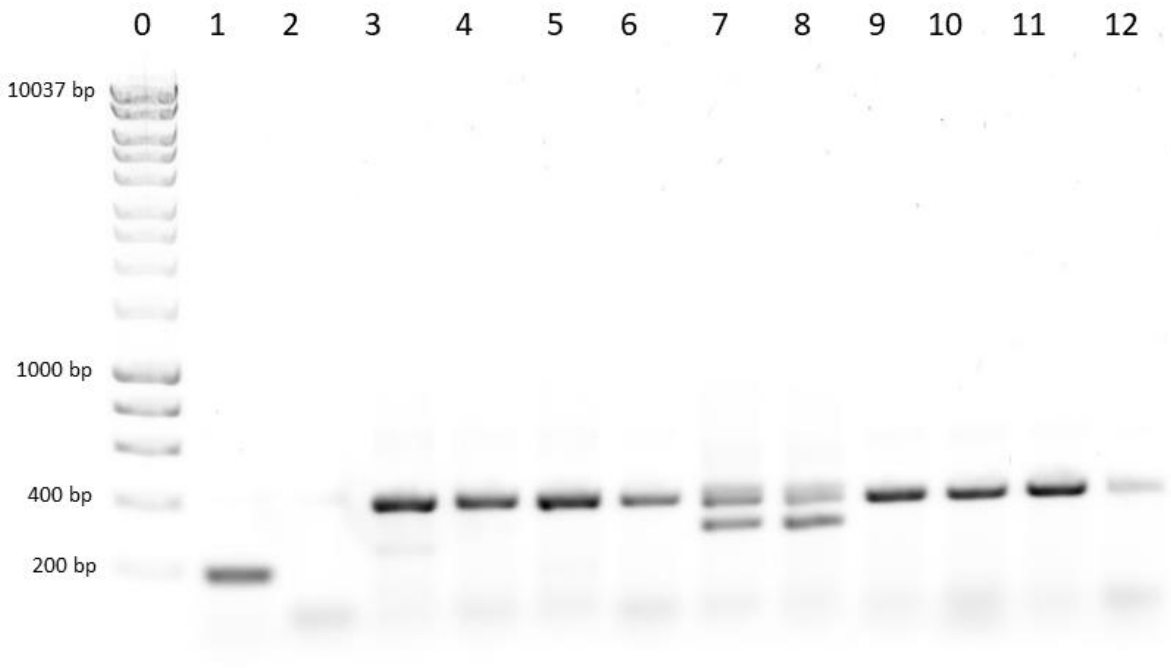

Figure 4-14 PCR products assessed using ‘Avramenko’ITS-2 primers and DNA extracted using CTAB (Lanes: 0: 1 kb Hyperladder 1: Positive control, 2: Negative control, 3: Widget pre, 4: Widget post, 5: Klettur pre, 6: Klettur post, 7: Major pre, 8: Major post, 9: Gertie pre, 10: Gertie post, 11: Seren pre, 12: Seren post).

4.4.10 Cloning and sequencing to confirm nematode DNA

The aim of this work was to check that the PCR was correctly amplifying nematode DNA, prior to preparing and sequencing it using the MinION. This was achieved by inserting the PCR amplicons into a vector and cloning using *E. coli*.

4.4.10.1 Cloning and sequencing: High Fidelity PCR

PCR was repeated using the optimised rDNA PCR conditions but using a high fidelity polymerase; MyFi 2 × mix polymerase (Bioline.com) to provide amplicons that could be inserted into a sequencing vector. PCR products were imaged using gel electrophoresis on a 1% w/v Agarose gel run at 100 V (Figure 4-15). A sample of DNA from another nematode was available, *Baylisascaris schroederi* and so this was included as a second positive control in addition to a second negative control of DNA from an equine faecal sample with a zero FEC.

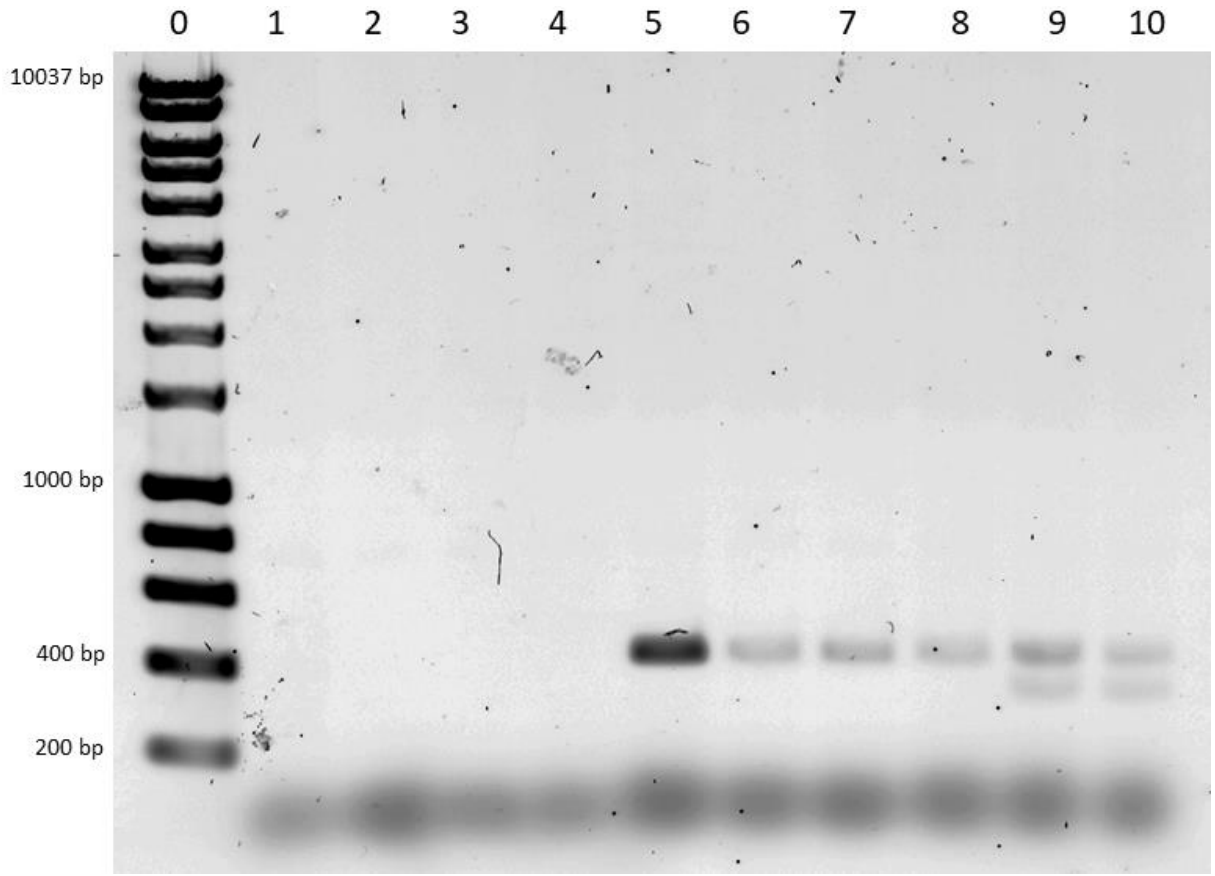

Figure 4-15 PCR products assessed using 'Avramenko'ITS-2 primers and high fidelity polymerase (Lanes: 0: 1 kb Hyperladder 1: Positive control (*P. redivivus*), 2: Positive control (*B. schroederi*), 3: Negative control, 4: Negative control Vinur zero FEC, 5: Widget pre, 6: Widget post, 7: Klettur pre, 8: Klettur post, 9: Major pre, 10: Major post)

The DNA samples extracted from Major (both pre and post; Figure 4-15 lanes 9 and 10) again showed a double band. All DNA extracted from the equine faecal samples amplified a product. Neither of the positive controls amplified anything likely due to low template DNA added (1 µl to be consistent with the faecal sample DNA; previously 4 µl of control DNA had been used).

Prior to vector insertion, selected amplicons were quantified using a NanoDrop spectrophotometer, with Widget pre giving 5.8 ng/µl (260/280 ratio of 1.87 and 260/230 ratio of 0.93) and Major pre showing 4.2 ng/µl (260/280 ratio of 2.15 and 260/230 ratio of 0.20).

4.4.10.2 Cloning and sequencing: Colony PCR product confirmation

PCR products from the overnight colonies transformed with PGEM Teasy containing the amplified PCR products were imaged using gel electrophoresis on a 1% w/v Agarose gel run at 100 V, to check that bands of the expected size were produced (Figure 4-16). It can be observed that all the Widget colonies (Lanes 1-8) were products of the same bp size, and that some of the Major colonies (Lanes 9-16) had cloned up the larger and the smaller of the two products that had been in the original PCR product.

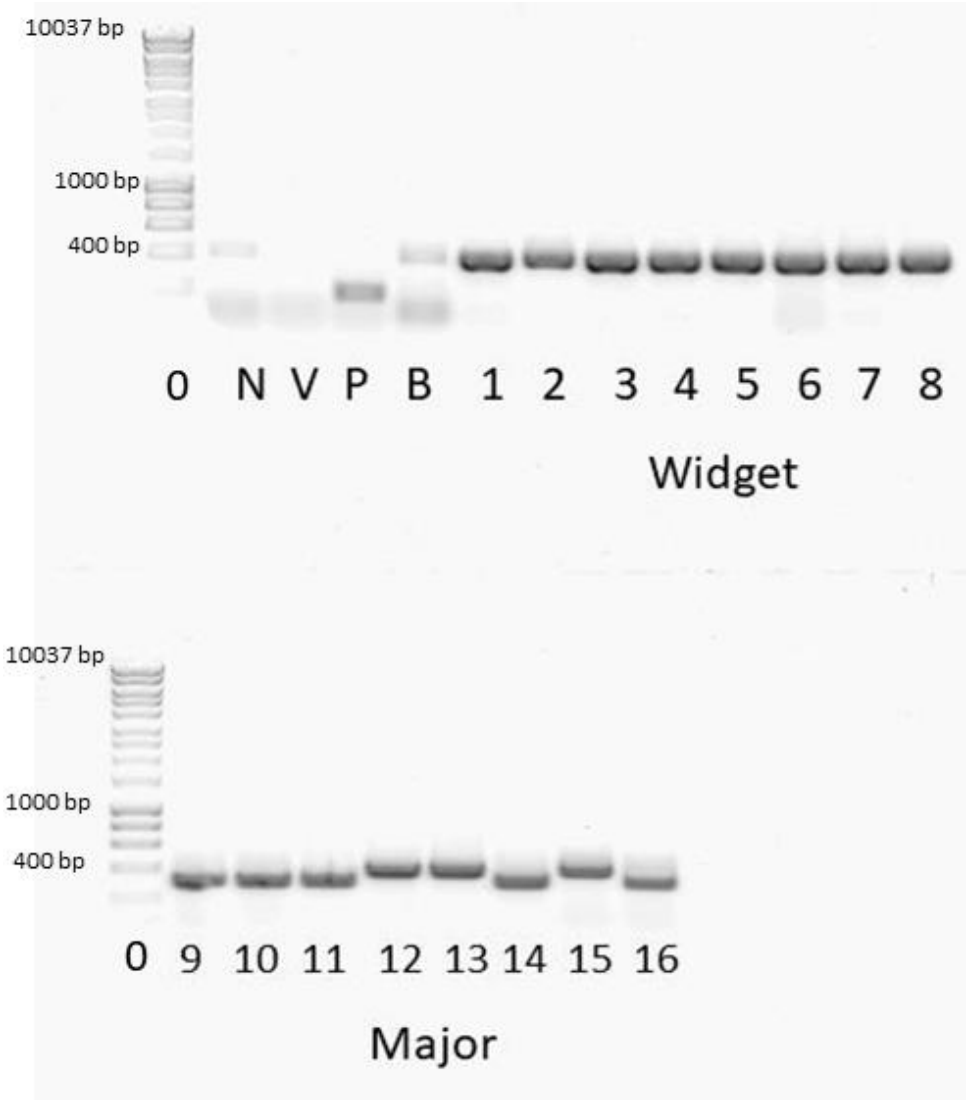

Figure 4-16 PCR products assessed from colony PCR using 'Avramenko' ITS-2 primers. (Lanes: 0: 1 kb Hyperladder, N: negative control, V: negative FEC control, P: positive control (*P. redivivus)*, B: positive control (*B. schroederi)*, 1-8: Widget samples, 9 – 16: Major samples)

Following colony PCR confirmation selected plasmids representing each product size were purified and quantified using the NanoDrop (Table 4-7). In total, between 200 and 250 ng of DNA was submitted for sequencing for each sample.

Table 4-7 NanoDrop quantification of Plasmid purifications prior to Sanger sequencing. Both 260/280 and 260/230 ratios are included for an indication of purity.

Sample	DNA ng / µl	260/280	260/230
6 (Widget)	48.2	1.68	1.41
11 (Major, small band)	310.7	1.84	2.25
12 Major, larger band)	89.6	1.81	2.12

4.4.10.3 Cloning and sequencing: colony growth and sequencing

The results of the Sanger sequencing were BLASTed using BLASTn against the National Center for Biotechnology Information (NCBI) database. Both forward and reverse samples for sample 11 (Major smaller band) and 12 (Major larger band) successfully sequenced, with the sample 11 giving a 98% sequence identity to the cyathostomin *Coronocyclus coronatus* and sample 12 giving a 100% sequence identity to another cyathostomin *Cylicostephanus goldi*. Unfortunately, sample 6 did not sequence successfully, with the forward sequence returning an error and the reverse sequence returning only a couple of bases.

4.4.11 Barcoding samples to enable multiplex analysis on MinION

In order to sequence multiple samples through the MinION (Oxford Nanopore Technologies, Oxford, UK), it was necessary to label each sample with a barcode. This would permit all of the samples to be sequenced simultaneously, with a separate output from the base calling for each sample that could be used to determine the species present in. Therefore, the aim of this work was to create a library of amplicons from each faecal sample, each labelled with a unique barcode.

4.4.11.1 Barcoding samples: barcode primer testing

Initially, three of the barcodes were tested, both directly with the DNA from faecal samples, and as a nested PCR on the PCR amplicons from these samples. Barcode one produced products from both DNA samples and PCR products (Figure 4-17a), whereas barcode 15 only produced products when used as nested PCR (Figure 4-17b). Barcode 35 also produced good products from the PCR products, and faint bands from the DNA samples (Figure 4-17c). However, there was some contamination in the negative control for barcode 35. Nested PCR was therefore chosen as the optimal method.

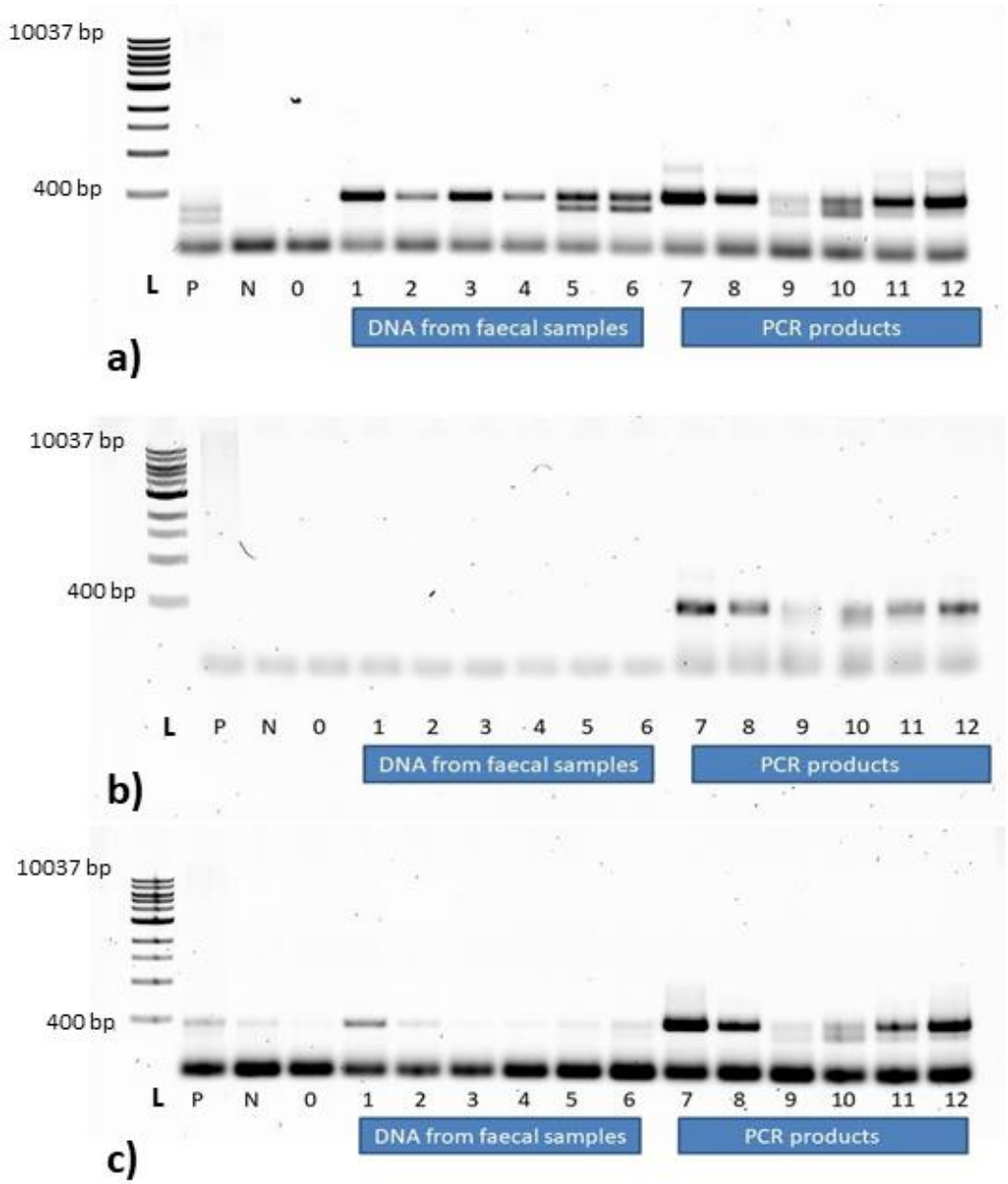

Figure 4-17 PCR products assessed using barcoded ITS-2 primers. (Lanes: L: 1 kb Hyperladder, P: Positive control (Schistosomule), N: Negative control, 0: Zero FEC negative control, 1 – 6: DNA from faecal samples used as template (Widget pre, Widget post, Klettur pre, Klettur post, Major pre, Major post), 7 – 12: PCR products used as template (Widget pre, Widget post, Yoyo pre, Yoyo post, Jigsaw pre, Jigsaw post)) a) Barcode 1, b) Barcode 15, c) Barcode 35

4.4.11.2 Barcoding samples: first nest of PCR

Amplicons of the ITS-2 rDNA region were produced from each DNA sample and run on a 1% w/v Agarose gel at 100 V (Figure 4-18) showing good bands for the majority of the faecal DNA samples. Five samples (13, 15, 16, 25 and 26) showed only faint products so a repeat PCR was performed on these samples (Figure 4-19). Samples 5 and 10 continued to be weaker than the other samples and so were also repeated. No band was observed for the positive control, possibly due to degradation of the template DNA. Given the PCR had worked for the other samples this was not considered important. No bands were observed for either the negative control or the zero FEC sample.

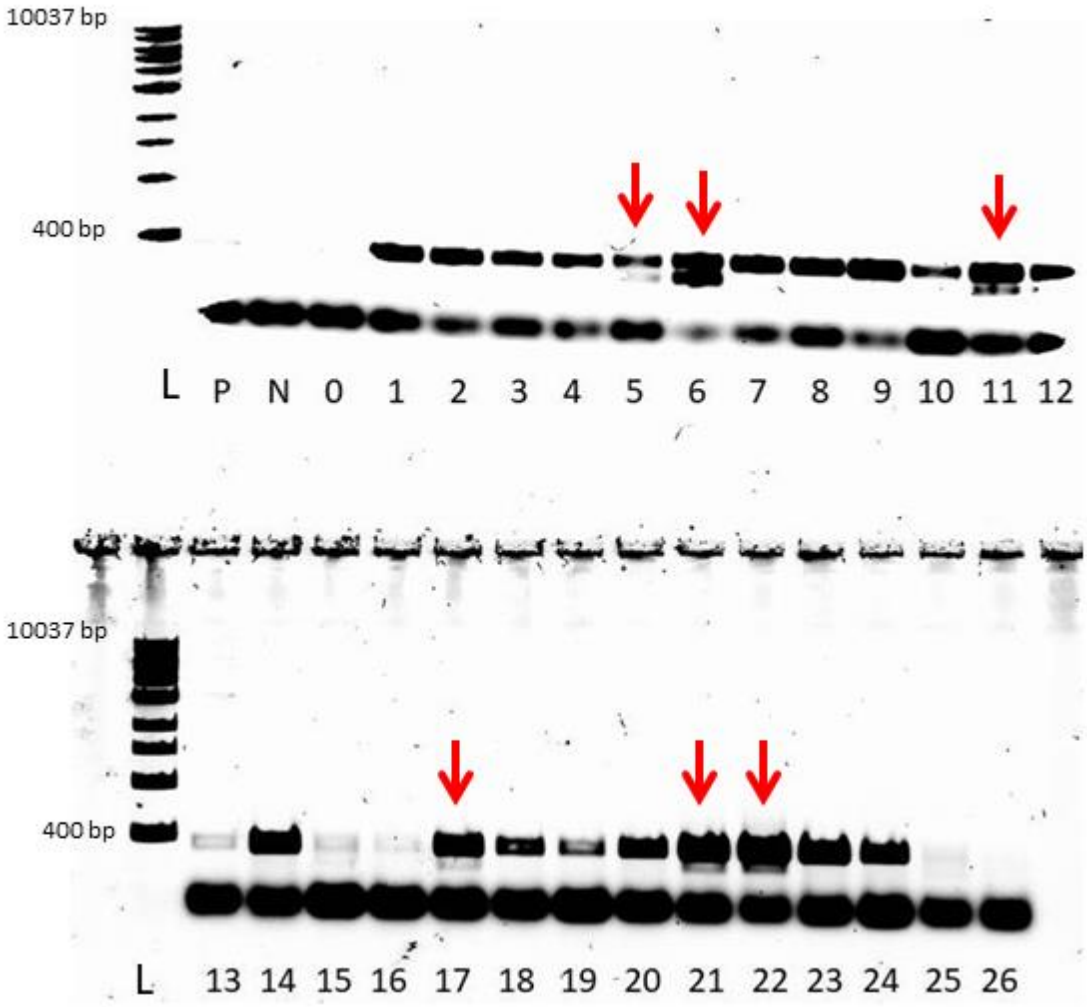

Figure 4-18 PCR products assessed using 'Avramenko' ITS-2 primers on all CTAB extracted faecal DNA samples (Lanes: L: 1kb Hyperladder P: Positive control (*P. redivivus*); N: Negative control (no template); 0: Negative control (zero FEC template); 1: Widget pre; 2: Widget post; 3: Klettur pre; 4: Klettur post; 5: Major pre; 6: Major post; 7: Gertie pre; 8: Gertie post; 9: Seren pre; 10: Seren post; 11:Jigsaw pre; 12: Jigsaw post; 13: Hope pre; 14: Hope post; 15: Billee pre; 16: Billee post; 17: Duke pre; 18: Duke post; 19: Sox pre; 20: Sox post; 21: Topaz pre; 22: Topaz post; 23: Mary pre; 24: Mary post; 25: Yoyo pre; 26: Yoyo post.) Samples producing double bands highlighted with arrows.

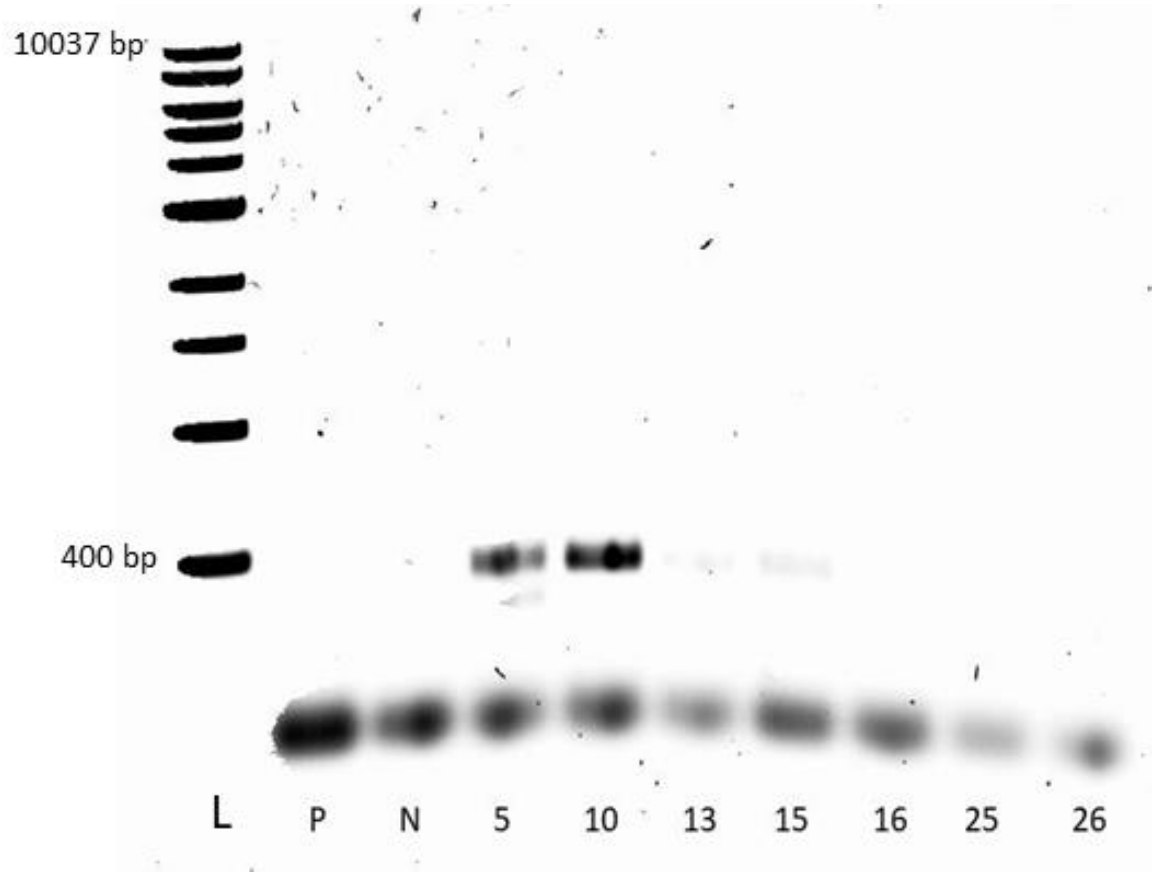

Figure 4-19 PCR products assessed using 'Avramenko' ITS-2 primers on CTAB extracted faecal DNA samples which had previously produced poor results. (Lanes: L: 1 kb Hyperladder, P: Positive control (*P. redivivus*); N: Negative control (no template); 5: Major pre; 10: Seren post; 13: Hope pre; 15: Billee pre; 16: Billee post; 25: Yoyo pre; 26: Yoyo post.)

Samples 5 (Major pre) and 10 (Seren post) produced good bands and the PCR products were retained to provide the templates for the barcoding. Those PCR products which did not produce a band were discarded. As the positive control still did not make a band, it was decided to try *S. mansoni* DNA as the positive control for the next attempt, and to use a different well in the PCR machine.

Figure 4-20 shows the third attempt at amplifying the final samples, with strong bands visible in samples 13 (Hope pre), 15 (Billee pre) and 26 (Yoyo post). Weak bands were visible in both versions of sample 16 (Billee post), suggesting that the

initial DNA extraction was the cause of the problem, not the dilution thereof. Sample 25 (Yoyo pre) still did not amplify well, and was discarded from future analysis. Samples 13a, 15a, 16a, 25a and 26a were all discarded due to possible contamination issues, having been included merely as a check to determine if there would be a difference in their performance.

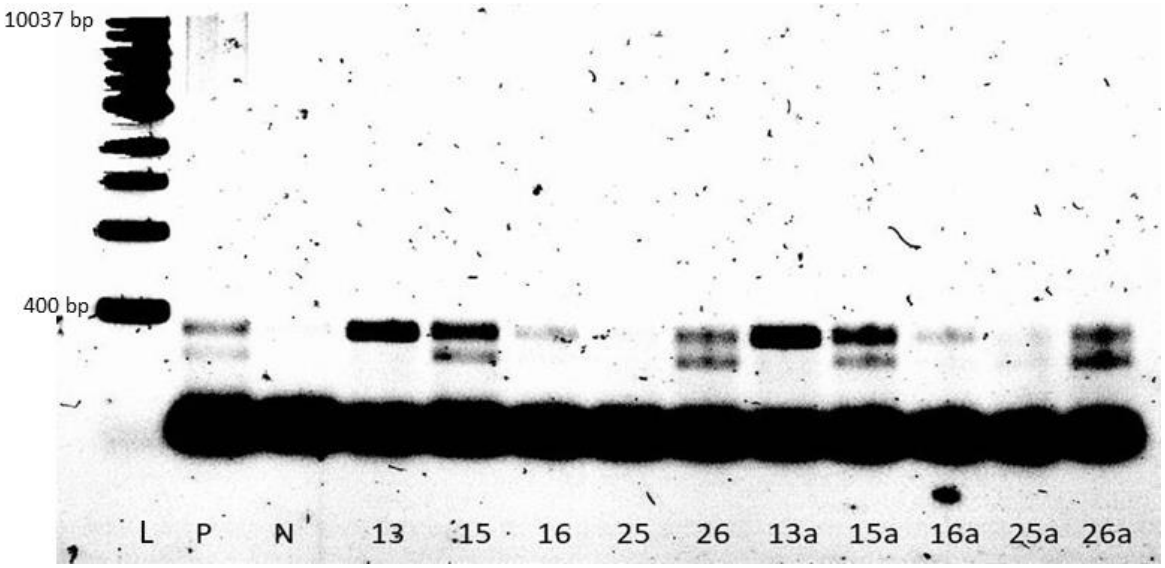

Figure 4-20 PCR products assessed using 'Avramenko' ITS-2 primers on CTAB extracted faecal DNA samples which had previously produced poor results, second repeat. (Lanes: L: 1 kb Hyperladder, P: Positive control (*S. mansoni*); N: Negative control (no template); 13: Hope pre; 15: Billee pre; 16: Billee post; 25: Yoyo pre; 26: Yoyo post, 13a – 26a same samples from a different DNA dilution.)

A final attempt a making the best possible template for the second nest of PCR to add the barcodes was performed for the poorest samples, and the products imaged using gel electrophoresis. The results were still fairly poor, and products were weak (Figure 4-21). Amplicons from 25a and 25b were pooled for the second nest.

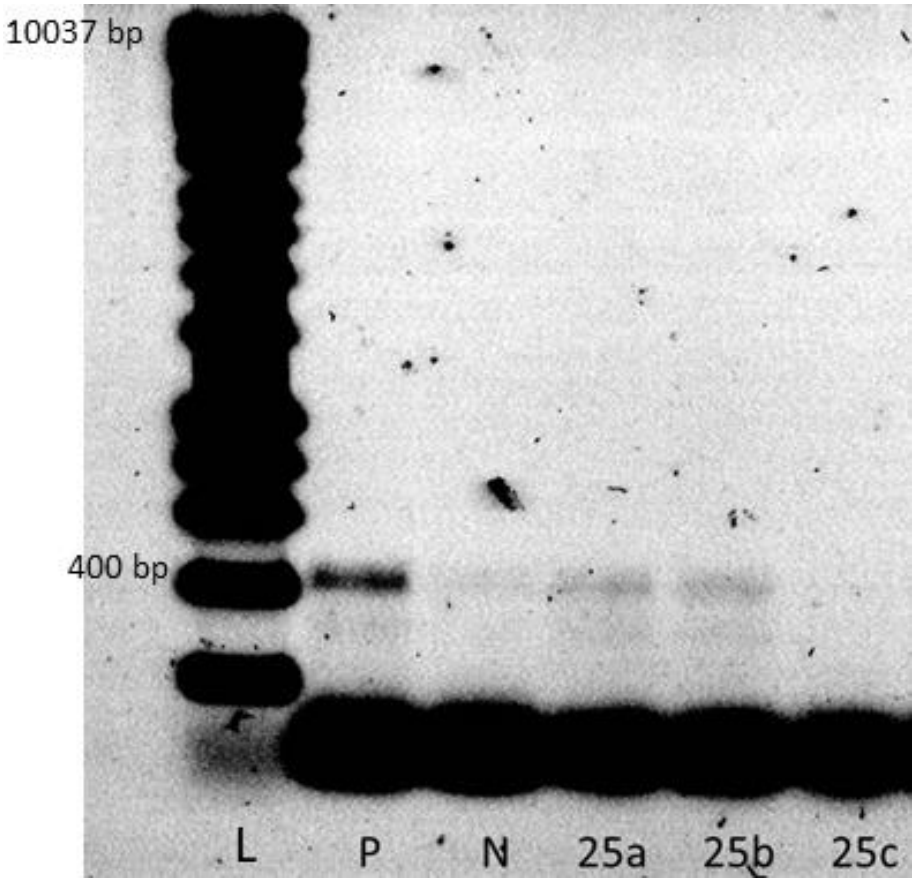

Figure 4-21 PCR products assessed using 'Avramenko' ITS-2 primers on CTAB extracted faecal DNA samples which had previously produced poor results, final repeat. (Lanes: L: 1 kb Hyperladder, P: positive control, N: negative control, 25a and 25b: Yoyo pre, 25c: Yoyo pre using 2 µl template)

4.4.11.3 Barcoding samples: second nest of PCR (adding barcodes)

Three replicates of the second nest of PCR were produced for each barcode, in order to provide sufficient product for sequencing using the MinION. Barcoded amplicons of the ITS-2 rDNA region were produced from each DNA sample and run on three 1% w/v Agarose gels at 100 V. Figure 4-22 is a representative example.

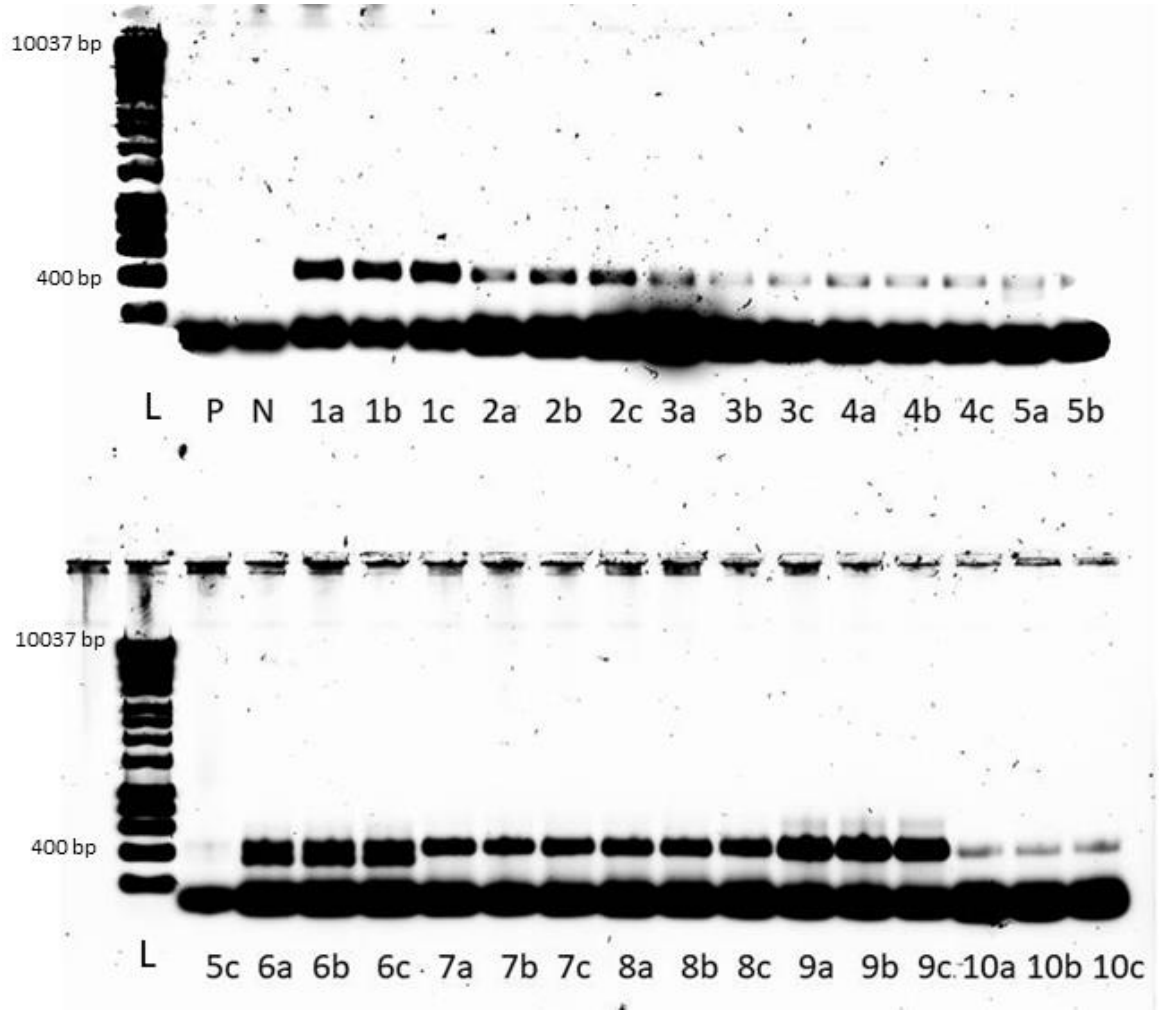

Figure 4-22 PCR products assessed using barcoded ITS-2 primers on CTAB extracted faecal DNA samples (Lanes: L: 1 kb Hyperladder, P: positive control, N: negative control, 1a, 1b, 1c: Widget pre, 2a, 2b, 2c: Widget post, 3a, 3b, 3c: Klettur pre, 4a, 4b, 4c: Klettur post, 5a, 5b, 5c: Major pre, 6a, 6b, 6c: Major post, 7a, 7b, 7c: Gertie pre, 8a, 8b, 8c: Gertie post, 9a, 9b, 9c: Seren pre, 10a, 10b, 10c: Seren post)

The second nest of PCR to add the barcodes was repeated for samples 13, 15, 16, 19, 25 and 26, and the results imaged on a gel (Figure 4-23). Four of the samples produced a better result on this second attempt (13, 15, 25 and 26), sample 16 was worse, and sample 19 was questionable.

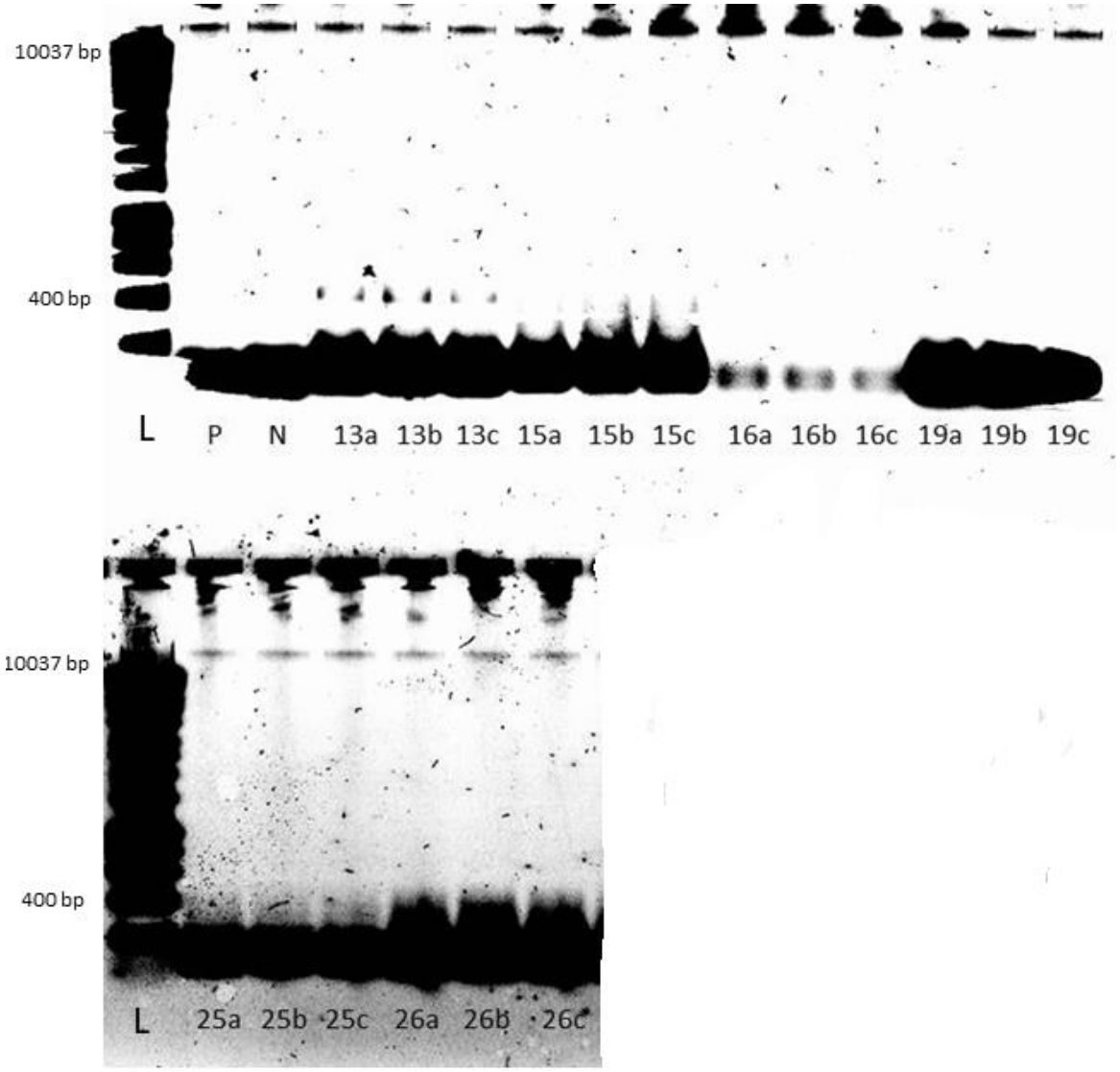

Figure 4-23 PCR products assessed using barcoded ITS-2 primers on CTAB extracted faecal DNA samples, repeat for samples with weak products (Lanes: L: 1 kb Hyperladder, P: positive control, N: negative control, 13a, 13b, 13c: Hope pre, 15a, 15b, 15c: Billee pre, 16a, 16b, 16c: Billee post, 19a, 19b, 19c: Sox pre, 25a, 25b, 25c: Yoyo pre, 26a, 26b, 26c: Yoyo post)

4.4.12 MinION sequencing of the equine nemabiomes

The aim of this section of work was to prepare the amplicons and sequence them using the Oxford Nanopore Technologies Ltd MinION device. The MinION sequences DNA by passing a single strand of DNA through a tiny pore in a membrane, and detecting the changes in electrical current that occur as each base

passes through. The sequencing was successful, and the results are discussed in section 4.4.13 Data analysis.

4.4.13 Data analysis

4.4.13.1 Data analysis: conversion of MinION output to DNA sequences

The raw output from the MinION produced 395,104 total reads, presented in FAST5 files. These data were run through Albacore (Oxford Nanopore Technologies Ltd) for base-calls with output files in FASTQ format. After base-calling, a total of 280,298 reads passed quality scores with 106,021 sequences failing quality scoring and 11,785 (< 3%) skipped (not processed concurrently due to lack of computer processing power).

The base-called reads from the pass folder were then run through PoreChop to identify and remove each barcode whilst binning each barcode into separate folders. The min_split_read_size was set to 400, meaning that if a read was found with a barcode in the centre, it would be split and fragments of less than 400 bp would be discarded. In total 173,011 reads were identified to a barcode and reads that weren't identified to a barcode totalled 66,775 (discarded 40,512). All barcodes were identified apart from barcode 16 (Table 4-8).

Table 4-8 PoreChop results sequencing ITS-2 amplicons produced from equine faecal samples, showing number of MinION reads identified to each barcoded sample by the PoreChop software. Barcodes 27 and 28 are zebra samples, to be discussed in Chapter 5.

Bar code	reads	Bar code	reads
1	10,690	**15**	1,253
2	6,870	**17**	3,912
3	2,463	**18**	2,872
4	3,222	**19**	1,254
5	3,046	**20**	4,524
6	8,052	**21**	13,436
7	9,119	**22**	20,405
8	8,167	**23**	10,573
9	22,407	**24**	3,310
10	2,754	**25**	547
11	9,737	**26**	1,712
12	3,243	**27**	1,110
13	2,040	**28**	1,226
14	15,067		
Total	**173,011**		
No barcode	**66,775**		
Grand total	**239,786**		

Sequences were analysed through CutAdapt to enable primers removal, filtered through a quality control and converted to FASTA format which could be BLASTed against the NCBI database to identify which species the sequences came from. In total, ten different runs were tried (section 4.3.15.1).

The results of the 10 different CutAdapt runs are shown in Table 4-9.

Table 4-9 CutAdapt results sequencing ITS-2 amplicons produced from equine faecal samples giving the total number of DNA sequences remaining for each barcode after different quality control filters Barcodes 27 and 28 are zebra samples, to be discussed in Chapter 5.

Bar code	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10
1	10,690	9,776	116	88	9,594	1,474	9,594	9,594	9,236	7,766
2	6,870	6,287	38	28	6,105	973	6,105	6,105	5,680	4,710
3	2,463	2,179	23	21	2,110	319	2,110	2,110	1,932	1,617
4	3,222	2,847	20	13	2,770	483	2,770	2,770	2,586	2,103
5	3,046	2,698	28	21	2,609	454	2,609	2,609	2,198	1,745
6	8,052	7,318	85	60	7,083	1,298	7,083	7,083	5,186	3,893
7	9,119	8,461	97	78	8,330	1,147	8,330	8,330	7,906	6,769
8	8,167	7,553	77	55	7,395	1,090	7,395	7,395	7,056	5,972
9	22,407	20,985	212	155	20,702	2,745	20,702	20,702	19,765	17,031
10	2,754	2,419	25	19	2,331	388	2,331	2,331	2,176	1,792
11	9,737	9,017	94	67	8,812	1,415	8,812	8,812	7,890	6,482
12	3,243	2,879	31	22	2,766	499	2,766	2,766	2,392	1,895
13	2,040	1,807	12	9	1,733	307	1,733	1,733	1,605	1,298
14	15,067	13,886	141	92	13,625	2,054	13,625	13,625	13,039	10,993
15	1,253	1,054	5	3	982	243	982	982	840	597
17	3,912	3,543	38	25	3,440	543	3,440	3,440	3,152	2,613
18	2,872	2,546	26	16	2,479	424	2,479	2,479	2,315	1,894
19	1,254	1,084	10	4	1,027	183	1,027	1,027	937	755
20	4,524	4,154	33	26	4,068	636	4,068	4,068	3,818	3,185
21	13,436	12,433	114	93	12,230	1,926	12,230	12,230	11,296	9,376
22	20,405	18,889	241	183	18,576	2,536	18,576	18,576	17,379	14,858
23	10,573	9,789	111	86	9,618	1,338	9,618	9,618	9,152	7,818
24	3,310	3,008	33	24	2,934	439	2,934	2,934	2,756	2,318
25	547	476	6	4	465	80	465	465	392	315
26	1,712	1,532	7	5	1,433	277	1,433	1,433	1,184	907
27	1,110	947	9	5	886	166	886	886	801	636
28	1,226	1,061	12	9	1,030	197	1,030	1,030	957	761
Total	173,011	158,628	1,644	1,211	155,133	23,634	155,133	155,133	143,626	120,099

Run ten removed both primers where they were found and kept only reads between 350 and 500 bases long. Run ten was judged to offer the greatest chance of discarding chimeric sequences and partial sequences, and returning only those

sequences that were a true amplification of the selected portion of the ITS-2 section of the rDNA gene.

4.4.13.2 Data analysis: BLASTn searches to identify species

The data from CutAdapt run 10 were compared against the NCBI database using a BLASTn search. It was decided to report only sequences with a certain percentage identical match, and three sensitivities were chosen – 97%, 95% and 90%. From these results, any with an alignment length of less than 35 bases (10% of the sequence length as per (Avramenko *et al*., 2015)) were discarded, as were any with e-values of 0.001 or greater. In order to compare the three sensitivities, a test barcode (barcode one) was used. Initially, 7,766 sequences were BLASTed against the database. At 97% identity over a length of 35 bases, only 41 sequences came up with a match. At 95% identity, 141 sequences were matched, and at 90% identity 2,608 came up with a match. It was decided to use the results of the 90% BLAST for further analysis.

Using the 90% sensitivity, it became apparent that most of the expected matches (i.e. cyathostomin species) had good, long alignment lengths of 300 – 350 bases. Given this, experimentation with discarding alignment lengths of under 100 bases to further increase accuracy was trialled on two barcodes (barcode 3 and barcode 8) (Table 4-10 and Table 4-11).

Table 4-10 Number of DNA sequence matches and proportion of sequences matched at >35 and >100 bases for barcode 3, BLASTn search of MinION data at 90% identity. Left two columns are actual number of sequences matched to each species, right two columns are % proportions of the total for each barcode that match to each species. Discard length is minimum length of sequence matched. Random species are those species which are not intestinal parasites of equines.

Species **Discard length**	**Barcode 3 - 35**	**Barcode 3 - 100**	**Proportions 35**	**Proportions 100**
Random species	34		7	
Coronocyclus coronatus	7	7	1	2
Coronocyclus labratus	1	1	0	0
Cyathostoma verrucosum	14		3	
Cyathostomum catinatum	135	135	27	30
Cyathostomum pateratum	22	21	4	5
Cylicocyclus ashworthi	2	3	0	1
Cylicocyclus auriculatus	1	1	0	0
Cylicocyclus nassatus	174	174	35	39
Cylicostephanus calicatus	3	1	1	0
Cylicostephanus goldi	70	68	14	15
Cylicostephanus longibursatus	32	31	6	7
Cylicostephanus minutus	1	1	0	0
Strongylus vulgaris	1		0	
	497	443	100	100

Table 4-11 Number of DNA sequence matches and proportion of sequences matched at >35 and >100 bases for barcode 8 BLASTn search of MinION data at 90% identity. Left two columns are actual number of sequences matched to each species, right two columns are % proportions of the total for each barcode that match to each species. Discard length is minimum length of sequence matched. Random species are those species which are not intestinal parasites of equines.

Species **Discard length**	**Barcode 8 - 35**	**Barcode 8 - 100**	**Proportions 35**	**Proportions 100**
Random species	80	1	4	0
Coronocyclus coronatus	12	5	1	0
Cyathostoma verrucosum	53		3	
Cyathostomum catinatum	100	99	5	5
Cyathostomum pateratum	30	30	1	2
Cylicocyclus auriculatus	1	1	0	0
Cylicocyclus brevicapsulatus	2		0	
Cylicocyclus elongatus	1		0	
Cylicocyclus insigne	3	1	0	0
Cylicocyclus leptostomus	2	1	0	0
Cylicocyclus nassatus	52	51	3	3
Cylicocyclus radiatus	4	3	0	0
Cylicostephanus calicatus	3	3	0	0
Cylicostephanus goldi	144	144	7	8
Cylicostephanus longibursatus	1551	1545	76	82
Cylicostephanus minutus	9	4	0	0
Cylicostephanus poculatus	6	2	0	0
Strongylus vulgaris	1		0	
	2054	1890	100	100

In total, for all barcodes, 35,209 sequences were matched at 90% identity over at least 100 bases, and 22 species of cyathostomin were identified, along with one large strongyle species namely *Craterostomum acuticaudatum*. There were only 23

matches which were not nematode parasites of horses, and these were three *Ancylostoma* species (hookworm parasites of dogs, cats and humans), two *Uncinaria* species (hookworm parasites of bears and sea lions) and three strongyle species that are parasites of marsupials. These may in fact have been novel equine species for which the ITS-2 sequence has not yet been characterised in the NCBI database. These 23 matches have been termed "Random species". The proportions of species identified in each barcode are depicted in Figure 4-24.

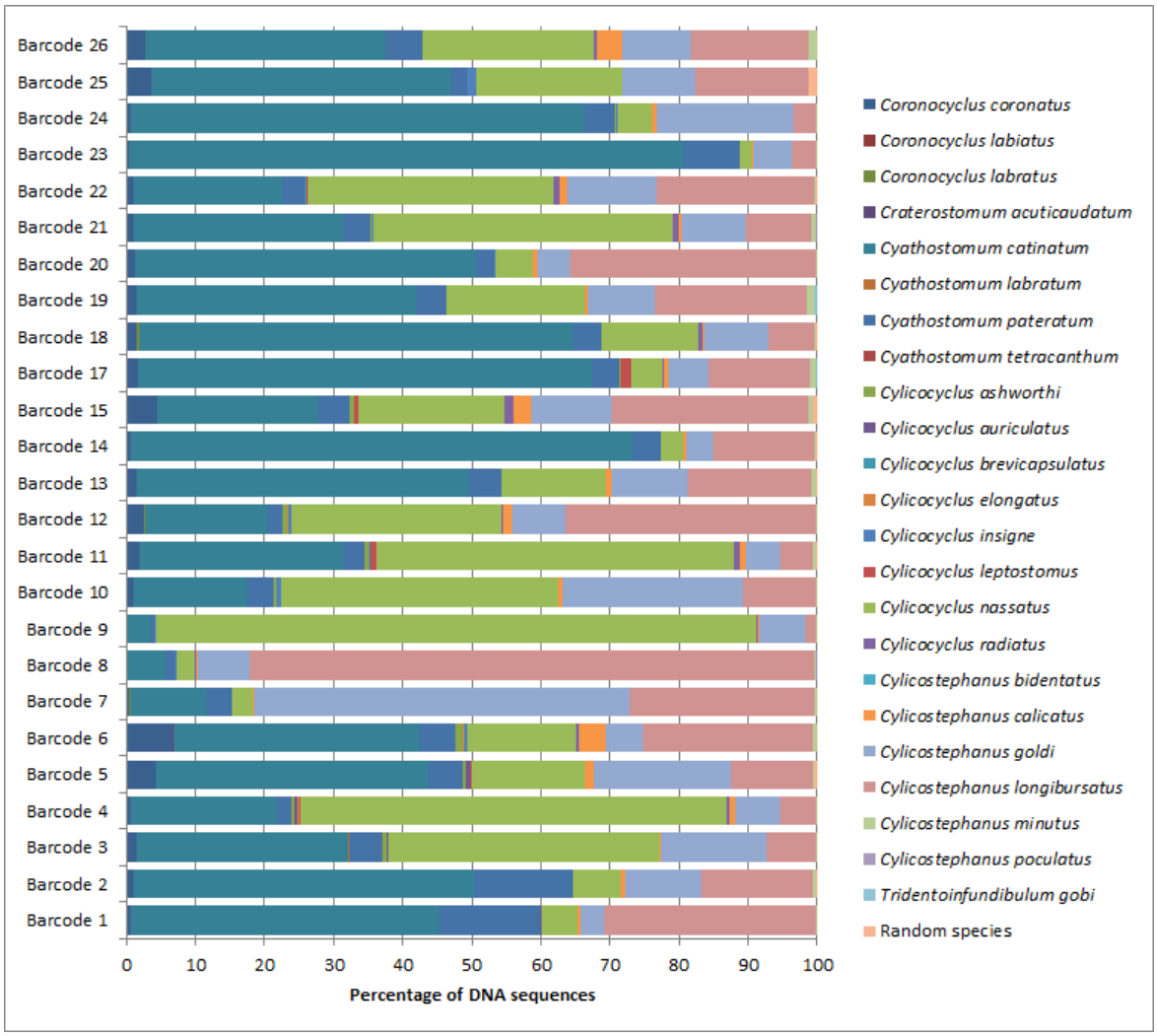

Figure 4-24 Parasite species identified from each barcoded equine faecal DNA sample by BLASTn search of NCBI database.

4.4.13.3 Data analysis: sequence proportion comparison

The species identified from each sample were quantified, and the proportions of each calculated. As the actual number of sequences produced in each sample was more a factor of the success of the DNA extraction than a reflection on the number of parasites present, from this point onward all calculations were performed on % proportions. Six principal species were identified namely *Coronocyclus coronatus, Cyathostomum catinatum, Cyathostomum pateratum, Cylicocyclus nassatus, Cylicostephanus goldi,* and *Cylicostephanus longibursatus*. Species which constituted less than 5% of the total of any sample were termed "Minor species" and were collected together for the purposes of data analysis. Box and whisker plots were produced to illustrate the species breakdown of sequences identified overall (Figure 4-25a), from pre-treatment samples (Figure 4-25b) and from post-treatment samples (Figure 4-25c). No major change in the overall species composition is noticeable from these plots.

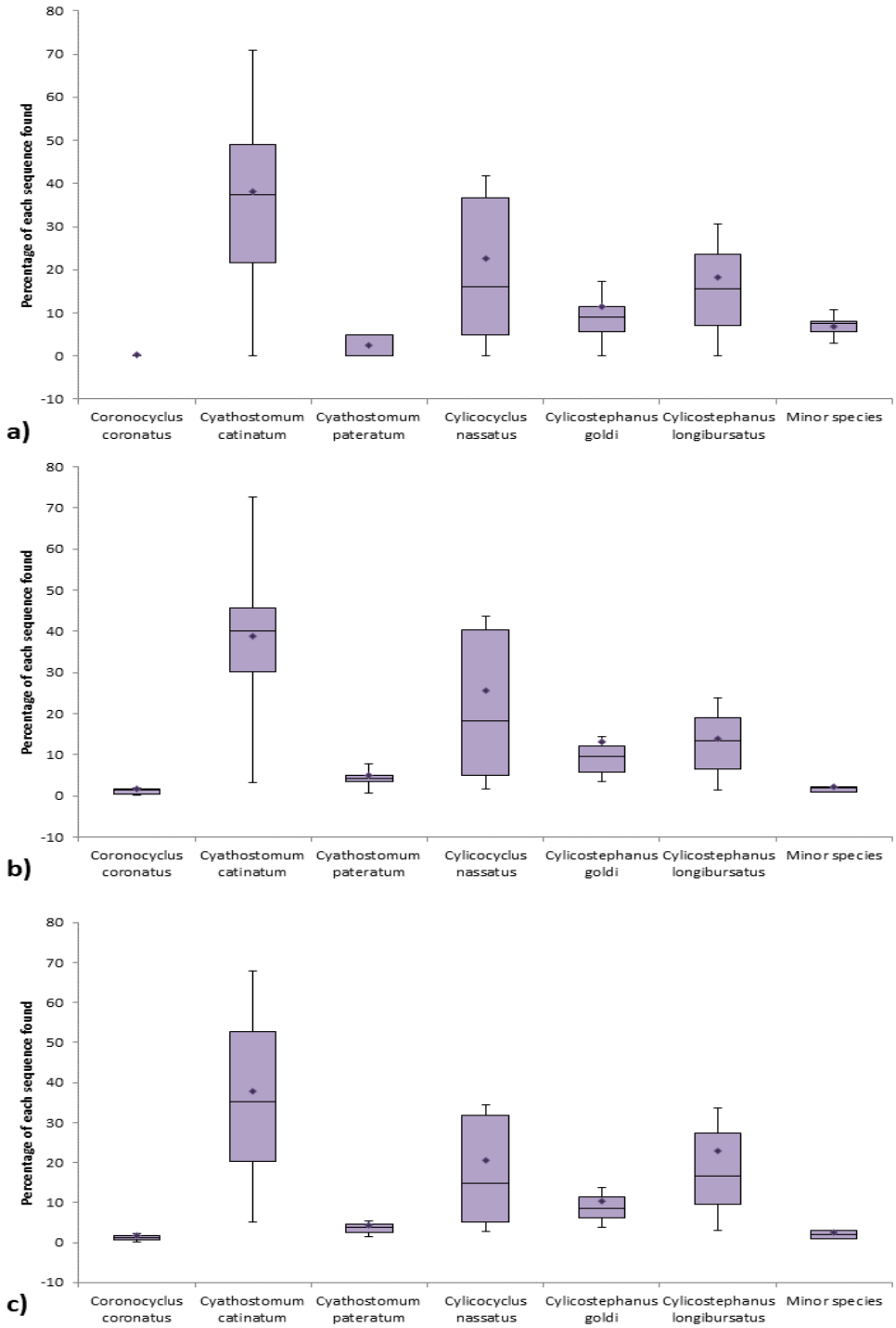

Figure 4-25 Box and whisker plot of helminth ITS-2 rDNA sequences identified from equine faecal samples a) total of all samples, b) samples from before BZ anthelmintic treatment, c) samples following BZ anthelmintic treatment.

Overall, the change in sequence proportion after BZ anthelmintic treatment demonstrated the greatest change in *Cylicostephanus longibursatus* which increased following treatment, and *Cylicocyclus nassatus* which reduced (Figure 4-26). However, this was not consistent over all the nemabiomes tested as can be observed in Figure 4-27 which demonstrates that all the principal species both increased and decreased in different populations following anthelmintic treatment.

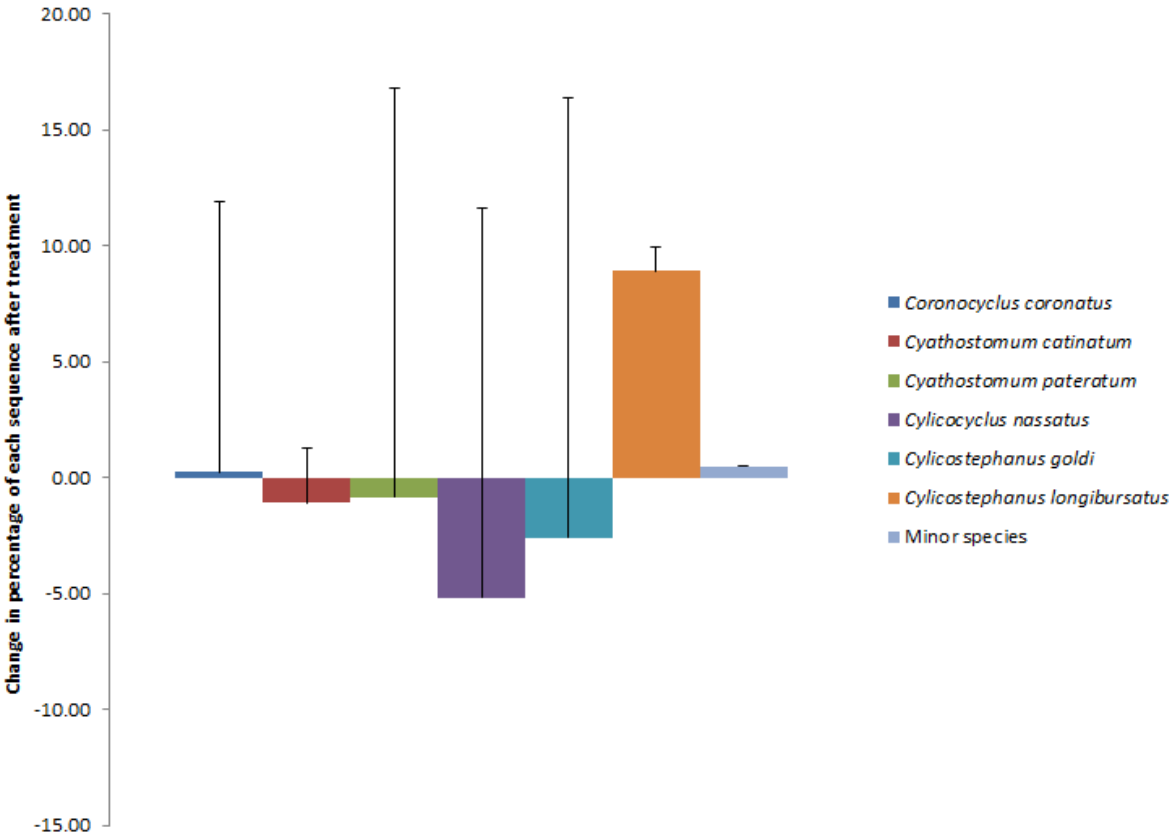

Figure 4-26 Overall change in sequence percentage of each helminth species identified from ITS-2 sequencing on faecal DNA samples following BZ anthelmintic treatment, data from all horses combined. Error bars show standard deviation.

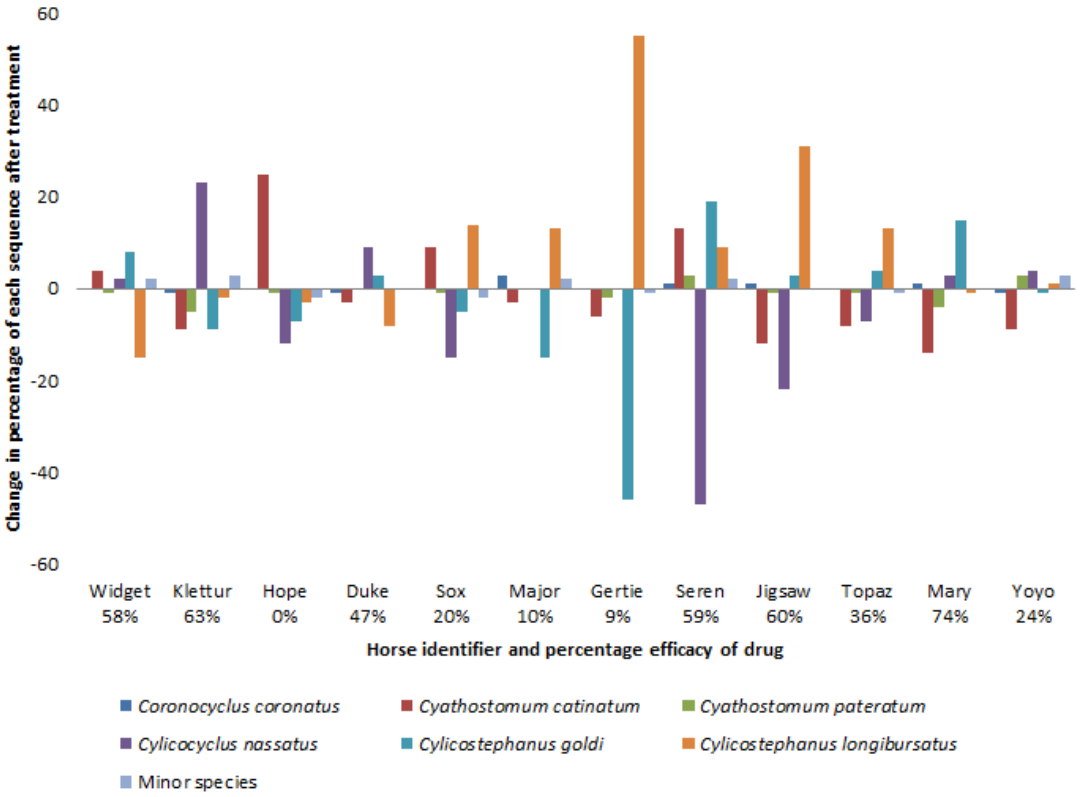

Figure 4-27 Individual change in sequence percentage of each helminth species identified from ITS-2 sequencing on faecal DNA samples following BZ anthelmintic, data from each horse shown separately, along with percentage anthelmintic efficacy

A fourth root transformation and Bray Curtis similarity analysis was performed on the sequence proportions, and visualised using an MDS plot; a two dimensional representation of the three dimensional change in sequence proportions (Figure 4-28). The calculated stress value of 0.11 demonstrates that the plot is a fair representation of the multi-dimensional data. The distances between each set of pre- and post-treatment points are similar (e.g. between points one and two, three and four etc.) which means that composition of sequences have changed by a similar amount, even though it is not always in the same direction. The analysis demonstrated that there was no significant difference at a community level between

pre- and post-treatment samples ($p = 0.79$), i.e. at a population level, BZ anthelmintic treatment had no effect on the community of parasites.

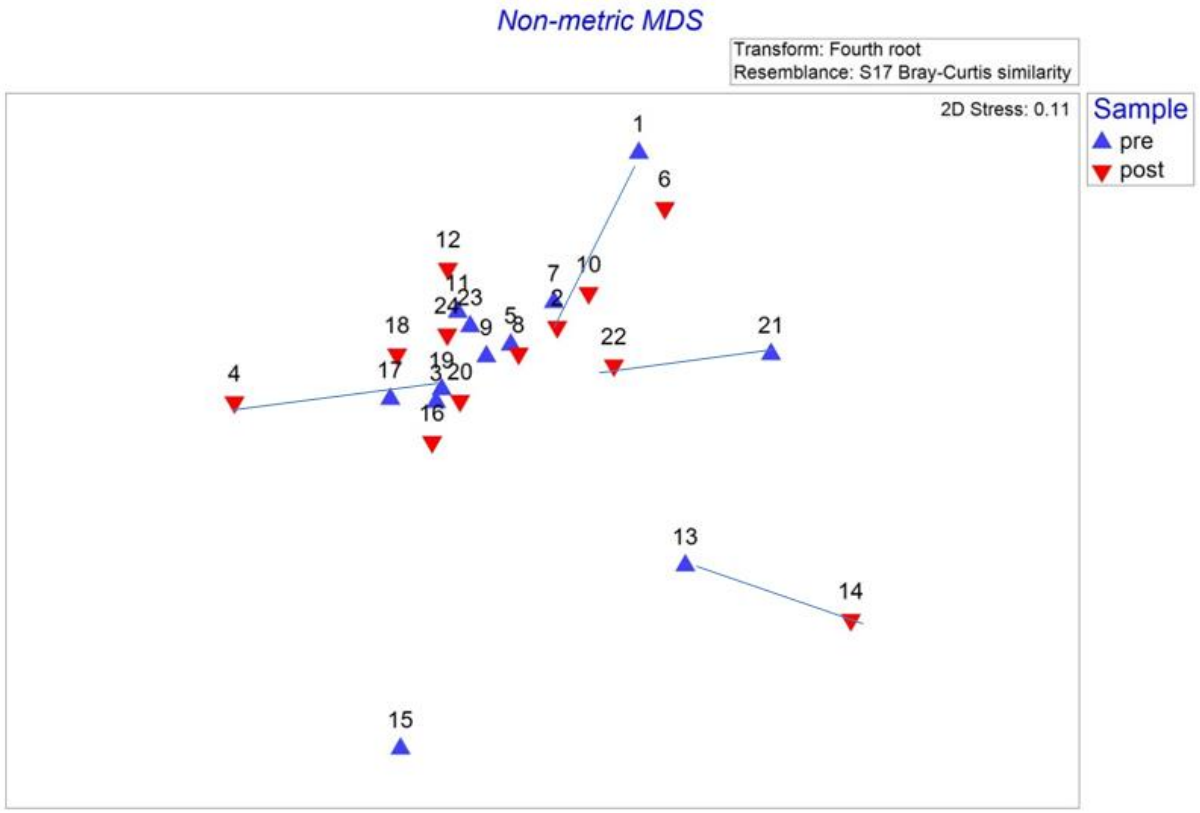

Figure 4-28 Non-metric MDS plot of diversity of pre- and post- BZ anthelmintic treatment sequence diversity in equine faecal samples, showing no significant difference at a community level between pre- and post-treatment samples. Representative pairs of samples (before and after BZ anthelmintic treatment on a single horse) have been linked with lines to illustrate.

4.4.13.4 Data analysis: effect of treatment on each principal species

Scatter plots were produced for each species, plotting post-treatment sequence proportions from each horse against pre-treatment sequence proportion. Trend lines were plotted, through the origin, to illustrate the percentage increase or decrease following treatment, and R^2 values added to indicate how strongly the relationship was explained by the data (Figure 4-29) with the data summarised in Table 4-12.

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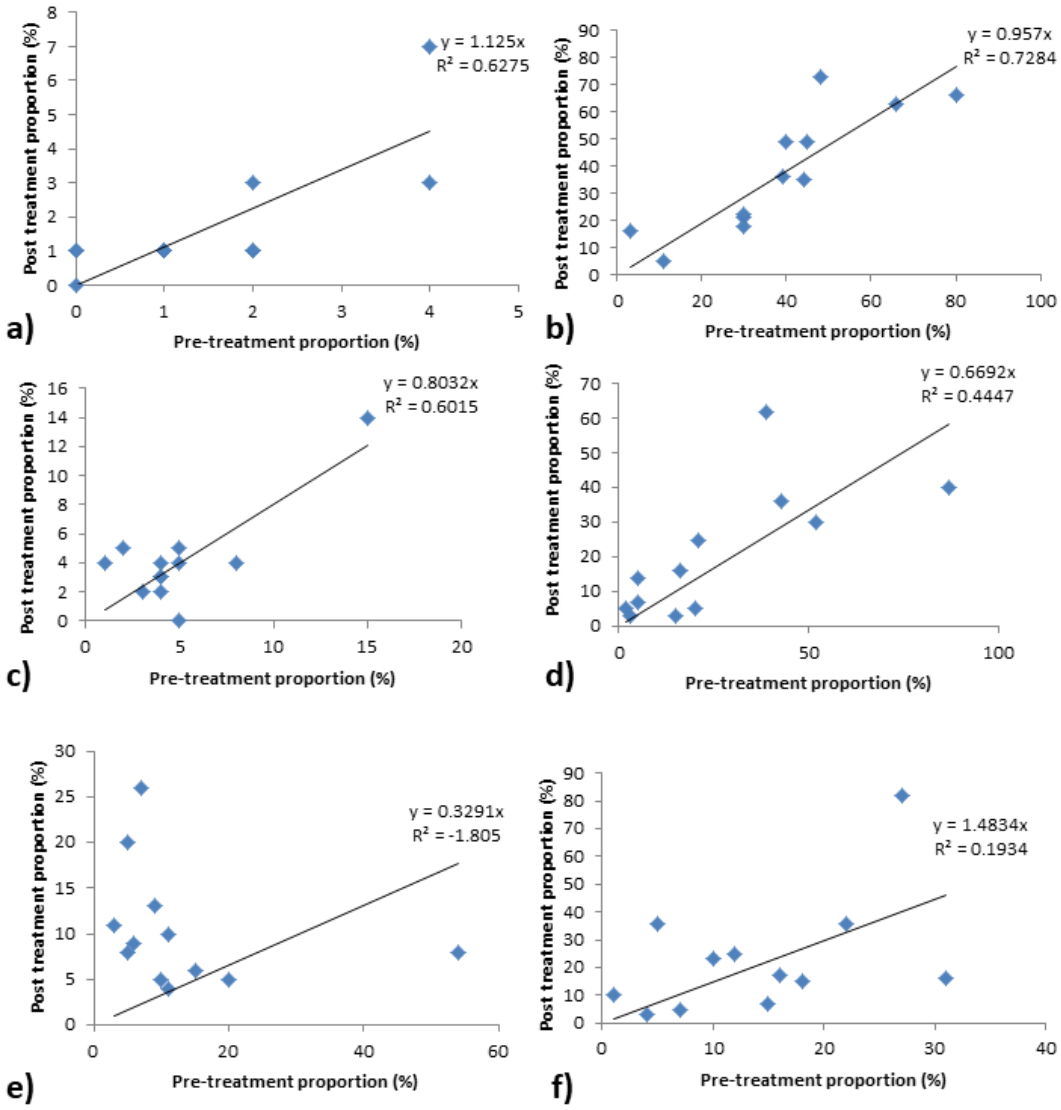

Figure 4-29 Scatter plots presenting the relationship between pre- and post-treatment DNA sequence proportions of each major helminth species present. Trend lines set through the origin. a) *Coronocyclus coronatus*, b) *Cyathostomum catinatum*, c) *Cyathostomum pateratum*, d) *Cylicocyclus nassatus*, e) *Cylicostephanus goldi*, f) *Cylicostephanus longibursatus*

Table 4-12 Summary of treatment effect on the proportions of sequences from the six principal species across all horse nemabiomes (listed order of prevalence), showing the percentage remaining after anthelmintic treatment (compared to the pre-treatment proportions), goodness of fit showing how consistent the change in proportion is for each species, and P values produced by Excel Regression data analysis function to show if this relationship is significant.

Species	Percentage of sequences pre-treatment	Percentage of sequences post-treatment	% of pre-treatment sequences remaining after treatment	Goodness of fit (R^2)	P value
Cyathostomum catinatum	39	38	96%	73%	0.0004
Cylicocyclus nassatus	26	21	67%	44%	0.0083
Cylicostephanus longibursatus	14	23	148%	19%	0.1140
Cylicostephanus goldi	13	10	33%	-18% (no correlation)	0.3876
Cyathostomum pateratum	5	4	80%	60%	0.0028
Coronocyclus coronatus	2	2	113%	63%	0.0020

Individual sample sequence proportions are shown in Figure 4-30.

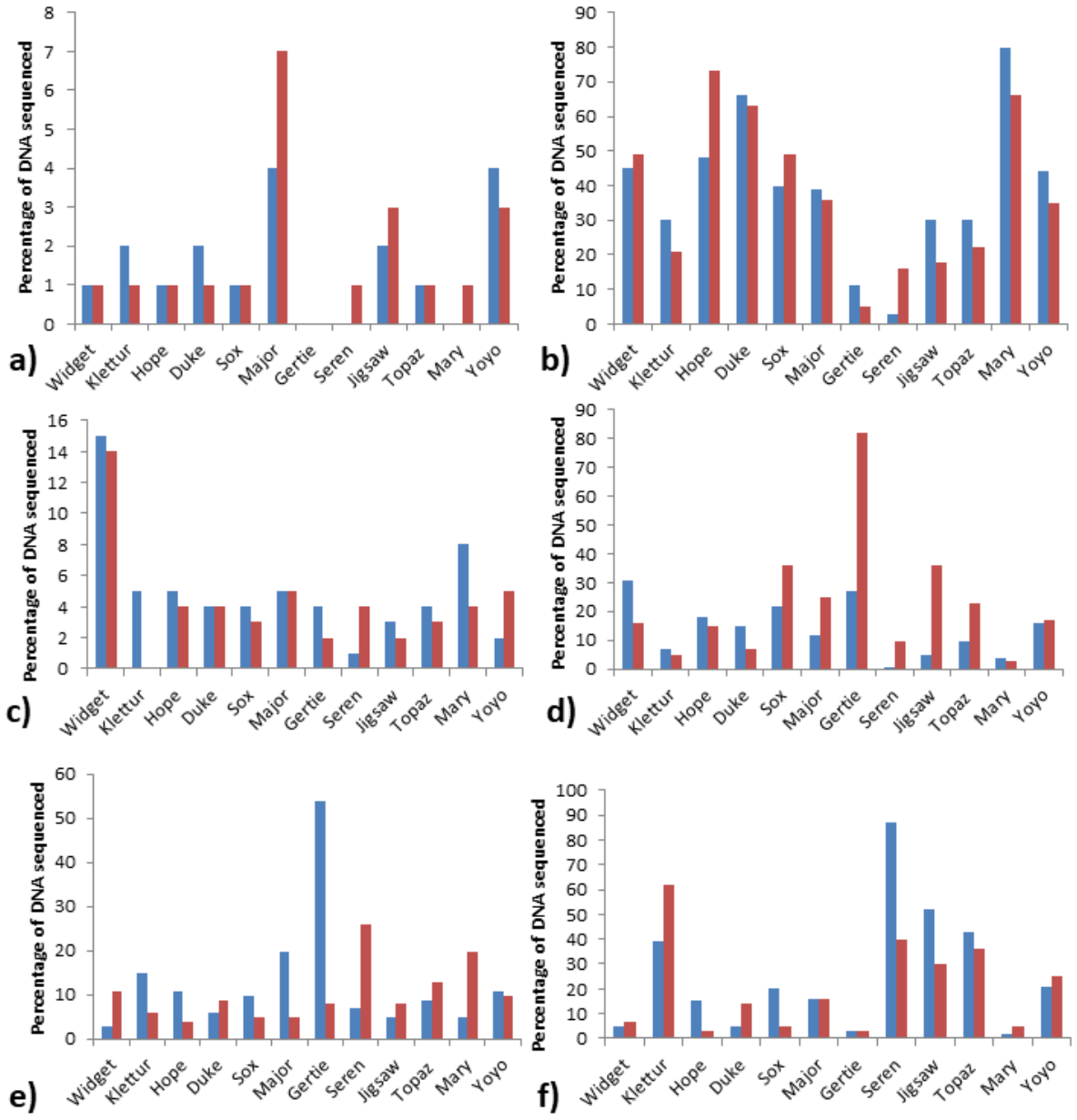

Figure 4-30 Proportions of ITS-2 DNA sequences found in equine faecal samples pre- and post- BZ anthelmintic treatment, for each individual horse. Blue bars show pre-treatment proportions, red bars show post-treatment proportions. a) *Coronocyclus coronatus*, b) *Cyathostomum catinatum*, c) *Cyathostomum pateratum*, d) *Cylicocyclus nassatus*, e) *Cylicostephanus goldi*, f) *Cylicostephanus longibursatus*

4.4.13.5 Data analysis: change in sequence proportion in relation to anthelmintic treatment efficacy

The change in sequence proportion was compared with the BZ anthelmintic treatment efficacy for the four species which showed a significant correlation between pre- and post-treatment levels. No significant correlation was observed between the change in prevalence of the DNA sequences for a species and the overall reduction in FEC after treatment in any case (Table 4-13), although there was a tendency towards significance for the percentage sequence change in the most numerous species, *C. catinatum.*

Table 4-13 Correlation between change in sequence proportion after treatment and drug efficacy for the four consistently resistant species – actual change in sequence proportions compared, and percentage change in sequence proportions. P values produced by Excel Regression data analysis function to show if this relationship is significant.

Species	**p value actual**	**p value percentage**
Cyathostomum catinatum	0.40	0.08
Coronocyclus coronatus	0.80	0.57
Cyathostomum pateratum	0.40	0.86
Cylicocyclus nassatus	0.98	0.10

4.4.13.6 Data analysis: differences between yards

Initially, it was hoped that a comparison could be made between the populations of helminth species observed at the three different sampling locations. However, due to the low levels of parasitic infections identified, there were insufficient samples tested at two of the locations for the results to be reproducible. Contingency tables were produced to investigate whether the pre- or post-treatment samples were significantly

different from each other in the three locations, and also whether they were significantly different from each other across all the samples (Table 4-14).

Table 4-14 Summary of contingency tables to show significant difference in DNA sequence proportions of the helminth species between samples in each yard. The principal species were used as the row variables, with the pre- or post-treatment sequence proportions as column variables.

Yard	Pre-treatment p value	Post-treatment p value
1	0.73	0.16
2	1.00	0.98
3	0.00	0.00
Overall	0.00	0.00

Overall, both pre- and post-treatment samples were significantly different from each other. Samples from yard 1 (n = 2) were not significantly different from each other either before or after treatment, neither were samples from yard 2 (n = 3). Samples from yard 3 (n = 7) were significantly different both before and after treatment. Sequence diversity proportions for each sample are shown in Figure 4-31.

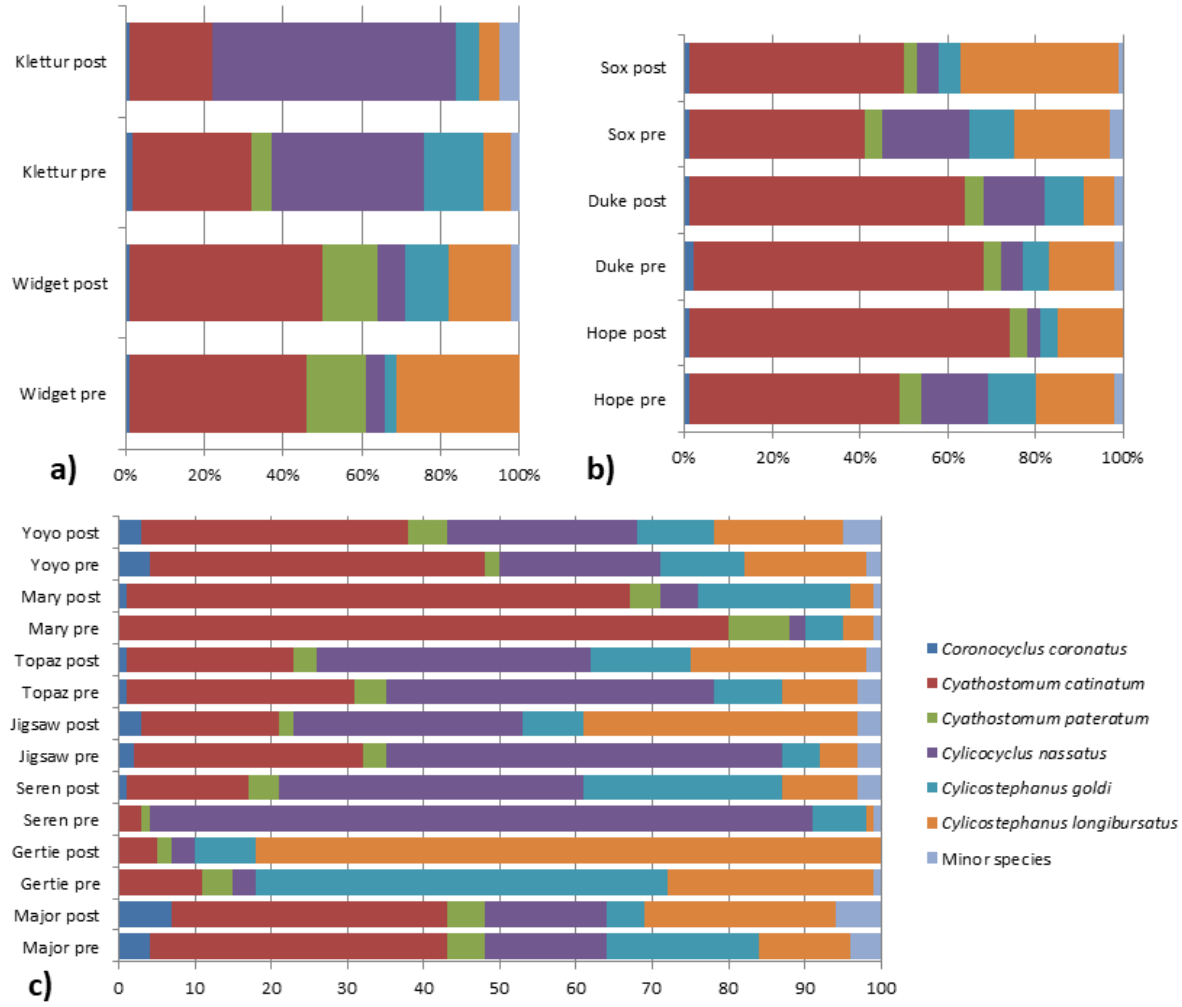

Figure 4-31 Proportions of ITS-2 DNA sequences from each helminth species in pre- and post- BZ anthelmintic treatment equine faecal samples a) Yard 1, b) Yard 2, c) Yard 3

4.4.13.7 Data analysis: species diversity and egg counts

The aim of this section of work was to see if there was a relationship between faecal egg counts and the diversity of species identified in the samples. This was performed by calculating Shannon indices for the pre- and post-treatment DNA sequences in each sample. The mean Shannon index for pre-treatment samples was 1.309 ± 0.321 and for post-treatment samples 1.343 ± 0.314. There was no significant correlation

between either pre- or post-treatment FEC and Shannon index, or between pre-treatment Shannon index and post-treatment Shannon index ($p < 0.05$) (Table 4-15).

Table 4-15 Shannon index of species diversity of DNA sequences in pre-treatment and post-treatment faecal samples compared to FECs. Overall there was no significant correlation between either pre- and post-treatment Shannon indices, or between pre-treatment or post-treatment Shannon index and FEC. P values calculated using Regression feature of Excel data analysis.

Horse	FEC Pre (epg)	Diversity Pre	FEC Post (epg)	Diversity Post
Widget	874	1.359	368	1.456
Klettur	478	1.502	179	1.219
Hope	307	1.465	414	0.958
Duke	216	1.243	115	1.245
Sox	294	1.544	235	1.222
Major	354	1.663	317	1.782
Gertie	446	1.223	405	0.747
Seren	511	0.573	212	1.527
Jigsaw	368	1.381	147	1.573
Topaz	1058	1.478	672	1.595
Mary	690	0.788	179	1.095
Yoyo	271	1.490	207	1.698
Mean	489	1.309	288	1.343
SD	260	0.321	158	0.314
p value (FEC vs Shannon index)	0.597		0.950	
p value (pre Shannon vs post Shannon)	0.531			

4.4.14 Primer design: beta tubulin

The second part of the molecular genetics analysis involved looking for SNPs associated with BZ resistance, in case any new ones could be discovered.

Primers selected from the literature for further investigation are provided in Table 4-16.

Table 4-16 Primers selected from the literature to amplify βtubulin gene in DNA extracted from equine faecal samples

Paper	Forward primer	Reverse primer	Size of product	Species	Annealing temp
Hodgkinson *et al.* (2008)	5'-AACGCAATCAATGTGTATTTCGC-3'	5'-GGTTTAATTACCCAAGTTTGAG-3'	Isotype 1 of beta tubulin gene - full length gene 1347bp	cyathostomins	63º
Ishii *et al.* (2017)	5′-GCTAACTCACTCACTTGGAGGA-3′	5′-CTTTGGTGAGGGAACAACG-3′	codon 167 120bp	cyathostomins	53º

4.4.15 PCR optimisation: β tubulin

With success in amplifying nematode DNA with rDNA primers an attempt was made to amplify the beta tubulin isotype 1 gene. This would enable the identification of polymorphisms in this gene that might either be existing known mutations, or potential novel ones.

4.4.15.1 PCR optimisation: beta tubulin Hodgkinson primers

The first primer set to be tested were those from Hodgkinson *et al.*(2008), which amplify the full length gene of isotype 1 of the beta tubulin gene. Hodgkinson had used these primers on cDNA made from RNA. The PCR products were imaged using gel electrophoresis however no bands were visible other than for the positive control (Data not shown).

As no bands were visible, the primer set was re-examined, but using different PCR conditions, as laid out in Hodgkinson *et al* (2008). The major difference was a longer extension time, to allow a larger product to be created. The PCR products were imaged using gel electrophoresis but again no bands were observed for any of the samples (Data not shown). The experiment was repeated again with an addition of increasing to a two minute extension time and finally a three minute extension time. In all cases no PCR products were amplified from the DNA from faecal samples even with a three minute extension time, and thus the primer set were discarded at this point (Data not shown).

4.4.15.2 PCR optimisation: beta tubulin Ishii primers

The second primer set to be trialled was that from Ishii *et al*. (2017), which amplifies codon 167 of the beta tubulin gene, one of the codons associated with BZ resistance

in cyathostomins (von Samson-Himmelstjerna *et al.*, 2007a). The PCR products were imaged using gel electrophoresis (Figure 4-32). As the expected product was small, approximately 120 bp, a low molecular weight ladder was also loaded onto the gel in addition to the regular 1 kb ladder used throughout the work thus far. No strong products were produced by this PCR, and multiple products were produced for both the positive control and several of the faecal DNA samples.

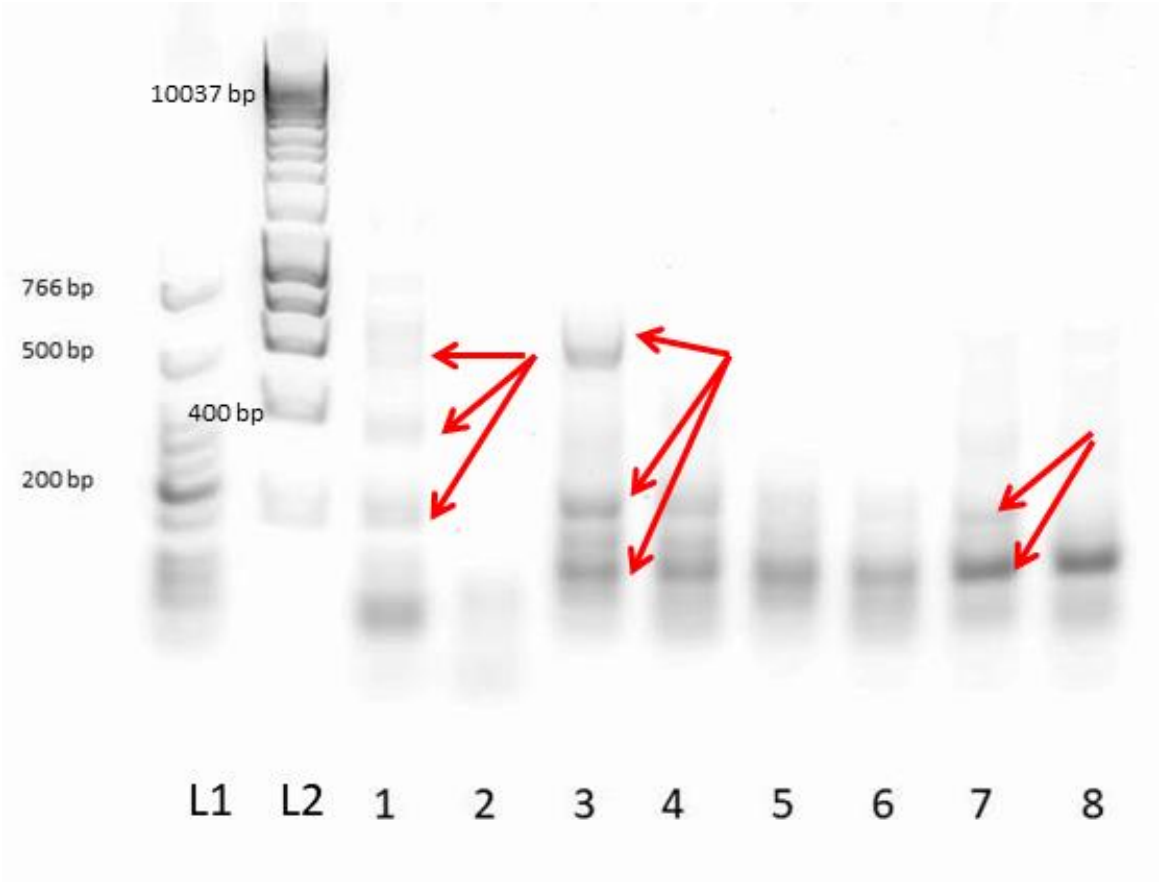

Figure 4-32 PCR products assessed from 'Ishii' β tubulin primers and DNA extracted from equine faecal samples both pre- and post- BZ anthelmintic treatment. (Lanes: L1: Low molecular weight ladder, L2 1kb Hyperladder, 1: Positive control (*P. redivivus*), 2: Negative control, 3: Widget pre, 4: Widget post, 5: Klettur pre, 6: Klettur post, 7: Major pre, 8: Major post) Multiple products highlighted with red arrows.

4.5 DISCUSSION

4.5.1 Resistance testing

This section of the work aimed to determine if there were populations of nematode parasites in any of the yards tested that were resistant to a class of anthelmintic. If resistant nematode populations were discovered, samples would be retained so that genetic analysis could be performed on both the pre-treatment and post-treatment samples to determine shifting equine nemabiomes.

For the yards tested, Ivermectin was observed to be fully effective 14 days after treatment. This was in accordance with the results found by Relf *et al.* (2014) and similar to Tzelos *et al.* (2017) where Ivermectin was effective in 17 out of 18 locations in the UK. The employed experimental design in the current work followed the current resistance testing guidelines published by (Coles *et al*., 2006). As with all anthelmintics, Ivermectin resistance will first be indicated by a reduced egg reappearance period (ERP) (Kaplan and Nielsen, 2010) and there is an indication that future resistance monitoring guidelines will recommend a post-treatment FEC at 28 days after treatment with Ivermectin (Coles, Pers. comm). Of concern was that an ERP after treatment with another ML, Moxidectin, showed a shortened ERP in seven out of eight premises tested in the UK (Tzelos *et al.*, 2017). Interestingly, in the current work eggs from *P. equorum* were observed post-treatment, suggesting reduced efficacy of Ivermectin against this parasite as reported previously (Beasley *et al.*, 2015; Bishop *et al.*, 2014; Boersema *et al.*, 2002; Hearn and Peregrine, 2003; Lind and Christensson, 2009; Lyons *et al.*, 2006; Lyons *et al.*, 2008; Näreaho *et al.*, 2011; Slocombe *et al.*, 2007; Veronesi *et al.*, 2010; Veronesi *et al.*, 2009). However,

and perhaps unfortunately, insufficient data were available for a significant conclusion to be drawn about *P. equorum* resistance in this study.

In all yards tested, Fenbendazole was found to have a reduced efficacy against cyathostomin egg shedding. The best post-treatment FEC reduction was 74% of the pre-treatment level, and post-treatment FEC in one horse increased. Therefore, in all animals the FECRT displayed resistance as defined by the WAAVP (Coles *et al.*, 1992). This is unsurprising, since widespread reports of BZ resistance in cyathostomins has led to it being contra-indicated as a means of control (Matthews, 2014). Despite this noted cyathostomin resistance, the one horse infected pre-treatment with *P. equorum* (at a level of 327 epg) showed a 100% reduction after treatment with Fenbendazole. Although there were not a statistically significant number of *P. equorum* infections to test, it does suggest that Fenbendazole (Panacur) still has a place in the anthelmintic arsenal. In addition, despite the well documented reduction in efficacy of BZ against adult cyathostomins, it has been observed that BZ has a 71.2% efficacy against encysted LL3/L4 larvae when administered at 10 mg/kg for five consecutive days, which was not significantly different from Moxidectin at 85.2% (Reinemeyer *et al.*, 2015).

4.5.2 Molecular genetics

All pre- and post- anthelmintic treatment samples were retained from this study for further molecular analysis in an aim to determine the nemabiome of the horses post BZ treatment. This would be done by next generation genetic sequencing of the samples collected both pre- and post-treatment, to determine the species composition. The samples would also be tested to determine if Single Nucleotide Polymorphisms (SNPs) were present that correlated with BZ resistance.

4.5.3 Primer selection: rDNA

In order to determine which species were present, a section of the small sub-unit ribosomal gene would be amplified using PCR. This gene is highly conserved among nematodes, and as the MinION is a powerful sequencer, it was decided to amplify the largest possible segment of this gene. Initial research suggested that the primers from Floyd *et al*. (2005) would be the best to use as they produced an amplicon of approximately 900 base pairs. However, the primers which worked the best, those that provided strong PCR products from faecal DNA extractions, were those from Avramenko *et al*. (2015) which had successfully been used in similar work by Mitchell *et al.* (2019). These produced amplicons of approximately 400 base pairs.

4.5.4 DNA Extraction

In order to determine which species were present in the pre- and post-treatment samples, and also to determine which SNPs were present in those species which had survived treatment with anthelmintics, it was necessary to extract the helminth DNA from the eggs contained in the equine faecal samples.

The DNA produced using the QiAmp DNA stool kit standard protocol yielded a higher quantity of DNA than that produced using the kit with an added bead beating step, when the test extractions were performed. It was therefore decided to not to incorporate a bead beating step when extracting the DNA from the faecal samples, for sequencing using the MinION. It is likely that the strongyle eggs in the faecal samples are more delicate and easier to lyse than alternative helminth species such as *Fasciola* eggs where bead beating is often incorporated (Dr Rhys Jones Pers. Comm.). Fragile nematode eggs is in agreement with earlier work, which demonstrated that *Trichuris* eggs were the only species examined that required

mechanical lysis, while strongyle eggs can be effectively lysed by a combination of freezing and heating (Demeler *et al.*, 2013).

Once the DNA extracted using the QiAgen kit was used as a PCR template, it became apparent that it did not contain a sufficient quantity of parasite DNA for the next step of the project, and thus a different DNA extraction method was explored.

Following the difficulties experienced trying to extract DNA using the QiAgen kit, an alternative method was assessed. Specifically, a CTAB method was adopted and the protocol chosen based upon and adapted from Dellaporta *et al*. (1983), Yu and Morrison (2004) and William *et al*. (2012). Initially, a comparison between freeze-dried and plain frozen sample was made, following earlier experimental work using freeze-dried samples. In this work it was discovered that freeze-drying merely reduced the weight but not the volume of the sample, and therefore did not permit a greater quantity of sample to be processed. However, the CTAB protocol stipulated freeze-dried material, so this was revisited. In fact, a far greater quantity of DNA was recovered directly from the frozen faecal sample than from the same sample that had first been freeze-dried. Thus, the freeze-drying step was omitted from future analysis.

Relatively good quantities of overall DNA were extracted using the CTAB method, although at this stage it was not known how much of this DNA was parasite DNA, microbial DNA, host horse DNA from sloughed off intestinal cells, or DNA from the grass that had been eaten (Tortora and Anagnostakos, 1987). A faecal sample from a horse with repeated zero FEC results was also used for DNA extraction, and this also yielded a high concentration of DNA, suggesting, as expected, that there was a large quantity of non-parasite DNA present in the samples. This zero-FEC DNA sample

would be used as an additional negative control during the PCR steps which were designed to separate out nematode DNA from the other DNA present in the samples.

4.5.5 DNA Extraction from Micro-I preparations

It was decided to determine if the samples prepared for imaging using the FECPAK^{G2} Cassette could be used for downstream DNA analysis. This would potentially enable an efficient workflow to detect and quantify a parasitic infection, and also determine the species present.

Initially, an attempt was made to remove the saline from the FECPAK^{G2} Cassette preparation. This was done by diluting the sample with water so that the eggs no longer floated, and then centrifuging the sample to collect the eggs. The NanoDrop results from the sample that was washed before extraction provided extremely low DNA levels possibly indicating that the DNA extraction had not been successful. It was possible that the eggs had been lost at the wash steps. Given that in the DNeasy protocol, one of the options when using cells was to re-suspend them in phosphate buffered saline (PBS), salt concentration was considered unlikely to disrupt the DNA extraction procedure and it was decided to repeat the experiment without washing the eggs from the saline to minimise egg loss. Despite this removal of wash steps, extremely low levels of DNA were again extracted.

To investigate if the salt was actually causing an issue with the extraction, or if washing the sample was losing the eggs, a fresh faecal sample was obtained (average infection level, FECPAK^{G2} slide count FEC showed 184 epg), and two FECPAK^{G2} Cassette preparations made. One preparation re-suspended the sediment from the sedimentation step in saline in the normal matter, and one re-suspended the sediment in water. An aliquot of the water-diluted sample was centrifuged to concentrate the

eggs, to see if this would improve the DNA extraction. As with previous results DNA extraction quantities and purity remained very poor. Thus, it was concluded that extracting DNA directly from the FECPAK^{G2} cassette preparation was unlikely to be successful with this approach.

As it was later discovered that the QiAgen kit yielded far poorer results in both quantity and quality for equine faecal extractions than the CTAB method of DNA extraction, it was decided to re-visit the question of whether DNA could be extracted directly from the FECPAK^{G2} Cassette preparation. As the salt was found not to affect the extraction of DNA, the CTAB protocol was followed using the solution as prepared for the FECPAK^{G2} Cassette but without a wash step. The outcome the two extractions performed were problematic and this DNA isolation pipeline was abandoned. One possible reason for the DNA isolation failure was the fact that the protocol to prepare the faecal samples for imaging in the FECPAK^{G2} Cassette involves diluting 23-fold the quantity of DNA available. Therefore, the numbers of eggs and hence the quantities of DNA extracted were low and not considered sufficient for the downstream application of nemabiome determination in this research, future work may allow sequencing of DNA extracted from the helminth eggs that were initially visualised using the FECPAK^{G2} Micro-I and determining the species of those eggs.

4.5.6 PCR optimisation: rDNA

In order to enable the species of nematodes contained in the samples to be determined, it was first necessary to optimise primers and PCR conditions for amplifying a segment of the small sub-unit ribosomal gene. Initial primer selection work demonstrated a strong product with the initial positive control (DNA from *B.*

glabrata, a snail) for both the primers taken from Marek *et al*. (2010) and Floyd *et al*. (2005). A weak band with the DNA from the faecal samples was shown with the primers taken from Peachey *et al*. (2017), so this was also selected for further investigation, although the product was very small at only 99 bp.

Initial work testing the primers against positive control DNA suggested that the 'Floyd' primers would be optimal, however, once PCR amplification of the final DNA from faecal samples was initiated, it the 'Floyd' primers failed to perform as well as expected, and so the decision was made to switch primers and use the ones which had been successful in similar work performed by Mitchell *et al*. (2019), which had been taken from Avramenko *et al*. (2015). These produced a smaller product than the 'Floyd' primers (400 bp instead of 900 bp) but proved to be more successful in producing amplicons from the DNA extracted from equine faecal samples.

4.5.7 PCR of DNA extracted from equine faecal samples

Once the 'Floyd' primers had been selected as the most promising ones to use, and faecal samples processed using the optimal method of extraction (frozen samples, without a bead disruption step), the samples were tested using PCR. These primers gave a band of approximately 900 bp from the positive control, which was DNA from *Panagrellus redivivus.* However, the PCR failed to produce any similar sized bands from any of the DNA extracted from faecal samples using the QiAgen kit. It was at this point that the decision was made to try an alternate method of DNA extraction – the CTAB method.

Initially, the 'Floyd' primers were again used, with 100 ng of template DNA. The PCR failed to amplify any products from all samples tested. One potential reason

could have been that too much template was used. If an excess of template DNA is included in a PCR reaction, there is the possibility that there will be insufficient primers to ensure that both the forward and reverse primers bind to the same strand of DNA. If this happens in the initial cycle of PCR, then there will not be any of the correct size of product to amplify in the further cycles, and the PCR will fail. In order to determine if this had been the problem, it was decided to repeat the PCR, but using only approximately 20 ng of template.

Using 20 ng of template and the 'Floyd' primer set PCR products were observed, so it was decided to try the primers used in Mitchell *et al*.(2019) and Avramenko *et al*.(2015) which had been taken from Gasser *et al*. (1993) on the diluted DNA samples. These primers gave a smaller expected product size, but had successfully been used to speciate cyathostomins in Mitchell *et al*. (2019) where they gave a product of approximately 400 bp. This product size differs from the size expected in other nematodes (250 bp when used on cattle nematodes in Avramenko *et al*.(2015), and interestingly the product produced in the positive control of *P. redivivus* was only 200 bp. These primers produced a band for every one of the samples tested, and thus it was decided to select these primers for future production of amplicons for sequencing and species identification. It was interesting that in one of the samples, two bands of slightly different sizes were produced, and speculation was that these were from different nematode species.

Prior to preparing and sequencing the amplified DNA from the equine faecal samples using the MinION, it was necessary to check that the PCR was correctly amplifying nematode DNA. This was achieved by inserting the amplicons into a

vector and cloning using *Escherichia. coli (E. coli)* which confirmed the amplification of cyathostomin DNA.

4.5.8 Barcoding and sequencing via MinION

In order to sequence multiple samples through the MinION (Oxford Nanopore Technologies, Oxford, UK) each sample was labelled with a unique barcode. In doing so, all of the faecal DNA samples could be sequenced simultaneously, with a separate output from the basecalling for each sample that could be used to determine the species present. Twenty six unique barcoded primers were used to label forward primers previously used in nemabiome analysis (Avramenko *et al.,* 2015), (Mitchell *et al.*, 2019) and the ITS-2 rDNA amplicons from the 13 paired pre- and post-treatment equine faecal samples were subjected to a second nest of PCR to label them with these barcodes. The MinION sequencing run was successful, and the data produced are discussed in section 4.5.9 Data analysis.

4.5.9 Data analysis

The data were passed through several quality control filters. Firstly they were base-called, and output which couldn't be identified to a base discarded. Secondly, the data were de-multiplexed and the reads separated into a folder for each barcode. The next stage was to remove the barcode primers, and discard DNA sequences that were either shorter or longer than the expected length. Several different filters were applied, in order to produce the highest quality data possible whilst still retaining sufficient sequences to produce meaningful results.

Run one was not set to discard any sequences; it only trimmed them to 250 bases. Run two discarded fragments under 100 bases, and did not discard many, suggesting that the majority of the sequences were longer than 100 bases. Runs three and four

discarded sequences if both primers were not located and actually discarded almost all of the data suggesting that both the entire primer sequences were not often present likely as a consequence of the barcodes being trimmed previously. Therefore, filters 3 and 4 were not used in subsequent runs. Run five discarded sequences less than 220 bases, and like filter 1 hardly discarded any sequences giving further evidence that the majority of sequences were longer than 220 bases. The suggestion of long sequence reads was encouraging, as short fragments would not be as accurate for determining from which species they came (Wommack *et al.*, 2008). Run six discarded sequences under 500 bases long, and was included to see if it would discard everything. In fact, over 23,000 sequences remained, meaning that some of the sequences were longer than expected. These sequences could possibly be chimeric sequences generated during the PCR (Corsaro and Venditti, 2018), and so it was decided to discard any reads longer than 500 bases. Run seven was set to trim the reads to 250 bases (the length of the amplicon expected as reported in Avramenko *et al.* (2015) and discard any reads shorter than 220 bases, and run eight was set to trim the reads at 400 bases, the length that the amplicon appeared to be following agarose electrophoresis in this study, and discard any reads shorter than 220 bases. These two runs discarded the same number of reads, along with run 5, as was expected. Run nine was the same as run eight, but discarded fragments less than 350 bases long. This didn't discard a huge amount more, meaning that most of the reads were at least 350 bases long – again encouraging, when the expected length was around 400 bases. The final run, run ten, removed both primers where they were found (and also anything before the forward primer or after the reverse primer), and kept only reads between 350 and 500 bases long. It was decided that filtering process 10 had the greatest chance of discarding chimeric sequences and partial sequences,

and returning only those sequences that were a true amplification of the selected portion of the small sub-unit rDNA gene. Filters were applied using CutAdapt (Martin, 2011) as in other nanopore sequencing research, such as Wei *et al*.(2018a; 2018b).

4.5.9.1 Data analysis: BLASTn searches to determine nemabiome

The data from CutAdapt run 10 were compared against the NCBI database (https://www.ncbi.nlm.nih.gov/) using a BLASTn search. It was decided to report only sequences with a certain percentage identical match, and three sensitivities were chosen – 97%, 95% and 90% similar to Mitchell *et al*. (2019). From these results, any with an alignment length of less than 35 bases (10% of the sequence length as per Avramenko *et al*. (2015)) were discarded, as were any with e-values of 0.001 or greater as per Wommack *et al.* (2008). It was decided to use the results from the 90% identity BLAST as per Avramenko *et al*. (2017) for two reasons. Firstly, very few matches were made at more than 90% identity, but more importantly, sequences from the higher identity were only short lengths (less than 100 bases), and it was thought more accurate to have a lower identity match over a good long sequence than a good match over a very short sequence.

In total, 35,209 sequences were matched at 90% identity over at least 100 bases, and 22 species of cyathostomins were identified, along with one large strongyle species, *C. acuticaudatum* which interestingly was present in only a post anthelmintic treatment sample. There were only 23 matches which were not nematode parasites of horses, and these were three *Ancylostoma* species (hookworm parasites of dogs, cats and humans), two *Uncinaria* species (hookworm parasites of bears and sea lions) and three strongyle species that are parasites of marsupials. This gave an error rate of

0.06%, or less than one sequence per sample as in Avramenko *et al*. (2017), which was considered to be very low. There is no evidence in the literature that hookworm infections are found in horses, so likely these 23 matches were equine parasite species which do not yet have their ITS-2 rDNA sequence characterised in the NCBI database, and the BLAST reported the closest match. Three species of cyathostomins (*Coronocyclus labiatus, Cyathostomum labratum* and *Tridentoinfundibulum gobi*) were present in only pre-treatment samples, although their occurrence was low. Their absence from post-treatment samples suggests that BZ anthelmintics may still be effective against these species and this may explain why they did not occur more frequently.

4.5.9.2 Nemabiome sequence proportion comparison

Six principal species were identified in equine nemabiomes pre and post BZ anthelmintic treatment; namely *Coronocyclus coronatus*, *Cyathostomum catinatum*, *Cyathostomum pateratum*, *Cylicocyclus nassatus*, *Cylicostephanus goldi*, and *Cylicostephanus longibursatus.* These identifications support the five principal species observed in (Traversa *et al.* (2009) with the addition of *C. coronatus.* Furthermore, all six species feature in the eleven principal species identified in horses from the Ukraine (Kuzmina and Kharchenko, 2008). However, only three species match the most abundant six species observed by Lind *et al*. (2003) which were *C. nassatus, C. catinatum* and *C. longibursatus*. These data suggest a relatively consistent equine nemabiome, given that there are at least 83 species of intestinal helminth parasites that infect horses (Lichtenfels *et al.,* 2008a). Species which made up less than 5% of the total of any sample were termed “Minor species” and were collected together for the purposes of data analysis.

Overall change in sequence proportion post BZ anthelmintic treatment demonstrated the greatest variability in *C. longibursatus*, which increased following BZ anthelmintic treatment, and *C. nassatus* which reduced in abundance. However, this was not consistent over all the equid nemabiomes examined as all the principal species variously increased and decreased in different populations following BZ treatment. Data analysis demonstrated that there was no significant difference at a community level between pre- and post-BZ treatment samples ($p = 0.79$). Thus, at a population level, BZ anthelmintic treatment had no effect on the community of parasites.

As there had been no significant effect of treatment observed when the horse was used as the experimental unit, it was decided to investigate the data from the point of view of each principal helminth species identified in the nemabiomes. This would determine if there was a relationship between the species and the effect of treatment.

Using the scatter plots produced, a significant correlation between pre- and post-treatment proportions was observed in four out of the six principal species ($p < 0.05$). The species which made up the greatest proportion of pre-treatment sequences, *C. catinatum* (39% of pre-treatment sequences) demonstrated limited response to BZ treatment, with 96% of sequences still remaining (goodness of fit 73% $p = 0.0004$), suggesting that this species was consistently BZ resistant across all the horses tested. The second highest proportion of sequences was *C. nassatus*, at 26% of pre-treatment sequences. This species demonstrated reduced BZ resistance (67% sequences remaining after treatment) and was also less consistent (goodness of fit 44%) yet still of significance ($p = 0.0083$). *C. pateratum* and *C. coronatus* were demonstrated consistently high levels of BZ resistance (80% remaining, goodness of

fit 60%, p = 0.0028 and 113% remaining, goodness of fit 63%, p = 0.002 respectively). However, these species represented only 5% and 2% of pre-treatment sequences. The remaining two species, *C. longibursatus* and *C. goldi*, did not exhibit consistent responses to BZ treatment with a goodness of fit of 19% (p = 0.1140) and -18% (p = 0.3876) respectively. These two species constituted 14% and 13% of pre-treatment sequences. Importantly, these data illustrate that BZ resistance is not global across all cyathostomin species.

It was interesting that the two species with an inconsistent response to treatment were both from the same genus, and also that they are found mostly in the dorsal colon – the most cranial (closest to the mouth) site that cyathostomins occupy (Morariu *et al.*, 2016). The species least well controlled by the administration of the Fenbendazole anthelmintic, *C. coronatus*, is located in the caecum which is the most caudal site occupied by cyathostomins (Morariu *et al.*, 2016). This fits in with the fact that BZ treatment is administered orally, and that Fenbendazole is poorly absorbed systemically (McKellar *et al.*, 2002) so its effect is mostly directly from the gut. The other three species consistently demonstrating levels of BZ resistance – *C. catinatum* (96% remaining after treatment), *C. nassatus* (67% remaining after treatment) and *C. pateratum* (80% remaining after treatment) - all principally occupy the ventral colon (Morariu *et al.*, 2016).To summarise, the location in the equine gastro-intestinal tract related to the response to BZ anthelmintic treatment with those cyathostomin species residing at the start of the tract being more well controlled than species residing further along. This is an important finding, as administration of sub-lethal concentrations of anthelmintics is known to lead to anthelmintic resistance (Sangster, 2001).

4.5.9.3 Change in nemabiome proportions in relation to BZ treatment efficacy

For those principal species which demonstrated consistent resistance to BZ anthelmintic treatment, no significant correlation between the total efficacy of BZ anthelmintic (i.e. the reduction in FEC following treatment for each horse) and the change in sequence proportion of the nemabiome principal species was observed. This was true whether the actual change in sequence proportion was used, or the percentage change. The two values were calculated in order to take into account the differing levels of each species – a change in sequence proportion from 5% to 10% would be more relevant than a change from 60% to 65% even though the actual change would still be 5%. This avoided over-stating the importance of those species that were not highly represented. This suggests that the reduction in FEC following anthelmintic treatment is not equal over all strongyle species, but varies with each horse owing to the significant differences in their nemabiomes.

4.5.9.4 Nemabiome differences between locations

Overall, both pre- and post-BZ anthelmintic treatment samples were significantly different from each other. Samples from yard 1 were not significantly different from each other either before ($p = 0.73$) or after treatment ($p = 0.16$), neither were samples from yard 2 ($p = 1.00$ before treatment, 0.98 after treatment). Samples from yard 3 were significantly different both before ($p < 0.001$) and after treatment ($p < 0.001$). It is not known whether this was due to the small number of samples (yard 1 $n = 2$, yard 2 $n = 3$, yard 3 $n = 7$) or because yard 3 was a rescue yard and therefore had a more transient population of horses than the other two yards.

4.5.9.5 Nemabiome species diversity and egg counts

It was decided to examine the data to determine if there was a relationship between faecal egg count and the diversity of species found in the samples. Species diversity was measured using a Shannon index. The mean Shannon index of both pre- and post-treatment samples was around 1.3, which is substantially lower than that observed previously in equids (Kuzmina and Kharchenko, 2008) and (Lind *et al.*, 2003). Kuzmina and Kharchenko (2008) reported Shannon indices of around 2.5 to 3 with (Lind *et al.*, 2003) reporting indices of around 2. However, (Kuzmina and Kharchenko, 2008; Young *et al.*, 1999) hypothesised that species diversity decreased in locations where regular anthelmintic treatments were applied, so a decade of anthelmintic treatment might have caused a reduction in the baseline species diversity. This reduced diversity is supported by the lower mean Shannon index of 1.8 found later by Sallé *et al.* (2018), and although that was still higher than observed in this study, the data had been collected in 2011 and 2012 so still follows the trend.

When linking diversity to FEC, no significant correlation ($p > 0.05$) was in fact observed between either Shannon index and FEC, or between the pre- and post-treatment Shannon indices for each sample. With this being the first study of its type, no similar studies were identified in the literature for a comparison to be made.

4.5.10 β Tubulin work

It had been hoped to identify the SNPs associated with BZ resistance, in case any new ones could be discovered. In order to do this, it was necessary to amplify the beta tubulin gene, which is the anthelmintic target for the BZ class of compounds

(Lacey, 1989, 1990). This would likely enable identification of mutations in the gene that might either be existing known mutations, or potential novel ones.

Firstly it was necessary to optimise primers that would amplify the β tubulin gene. It was decided to use the primers from Hodgkinson *et al.* (2008) as they amplify the whole of the β tublin gene, whilst also to try the primers from Ishii *et al.*(2017). A third set of primers, from Coles *et al.*(2006) and Pape *et al.* (2003) were not used, as they were designed to amplify separately the susceptible and resistant isotypes, and this would not be necessary when the products would be sequenced using the MinION.

The first of the two potential primer sets identified in section 4.3.7 to be tested were those taken from Hodgkinson *et al.* (2008). These had been used to amplify isotype 1 of the full length β tubulin gene in cyathostomins. Initially, the PCR was unsuccessful, so the extension time was lengthened. In the original paper, RNA was extracted from adult worms and used to make cDNA for the template. In the current research, gDNA was used as the PCR template; therefore the expected amplified product may have been longer than the 1437 bp in the original paper, due to non-coding intronic regions also being present. A longer extension time would therefore allow for a longer product to be produced, so extension time was increased from 10 seconds to one minute, which was the length of time used in Hodgkinson *et al.*, (2008). However, this approach was still unsuccessful, as were two and three minute extension times.

Having failed to amplify the entire β tubulin gene, it was decided to try the primers from Ishii *et al.* (2017), which amplify a region encompassing codon 167, one of the two codons associated with BZ resistance in cyathostomins (Lacey, 1989). As this

primer set would produce a smaller product it was considered inferior to the 'Hodgkinson' primers in relation to future nanopore sequencing, but would possibly be easier to amplify. The PCR produced some unclear products at around 50 bp and, since the expected product was around 250 bp, it was considered that this had not been successful.

As the literature dealt only with DNA extracted from helminth samples themselves and not from faecal samples, at this point it was decided that the available time would be better spent attempting to determine the nemabiome by sequencing the ITS-2 rDNA amplicons and analysing the results obtained from this work, and so the β tubulin work was put aside. It is possible that new primers could be designed for amplifying cyathostomin β tubulin genes from gDNA in the future; however this is hampered by the lack of quality genome data available. To date, only three equine parasite genomes are available in WormBase (https://parasite.wormbase.org/species.html) (*C. goldi, P. equorum and S. vulgaris*) and all three have very poor coverage. Time constraints did not permit further exploration of this avenue.

4.6 CONCLUSION

The work successfully determined the equine nemabiome from DNA extracted from faecal samples. Twenty two species of cyathostomin were identified, along with one large strongyle species namely *Craterostomum acuticaudatum* which was present in one sample, interestingly only after anthelmintic treatment. Six principal species were identified namely *Coronocyclus coronatus, Cyathostomum catinatum, Cyathostomum pateratum, Cylicocyclus nassatus, Cylicostephanus goldi,* and *Cylicostephanus longibursatus*. Three species were absent from all post-treatment

samples (*Coronocyclus labiatus*, *Cyathostomum labratum* and *Tridentoinfundibulum gobi*), although their overall occurrence was low. Their absence from post-treatment samples suggests that BZ anthelmintics may still be effective against these species and this may explain why they did not occur more frequently.

Initial comparison of the sequence proportions of the pre- and post-treatment samples showed that nemabiome populations responded very differently to BZ treatment. Two different effects were in operation: in some species, such as *Cyathostomum catinatum* and *Coronocyclus coronatus*, there was a high and consistent level of drug resistance, whereas in others BZ treatment had a more variable effect. As the most numerous species found both before and after treatment was *C. catinatum*, this partially explained the lack of difference between pre- and post-treatment samples. Failure of BZ treatment became greater in species which tend to occupy niches further along the digestive tract. The two species which did not show a consistent response to BZ tend to reside in the dorsal colon, the most cranial part of the tract occupied by cyathostomins. The species which was least well controlled by the administration of Fenbendazole, *C. coronatus*, tends to reside in the caecum, which is the most caudal part of the tract occupied by cyathostomins. This fits in with the fact that drug treatment is administered orally, and that Fenbendazole is poorly absorbed systemically (McKellar *et al.*, 2002) thus its effect is mostly directly from the gut.

No significant correlation ($p > 0.05$) was in fact found between either Shannon index of species diversity and FEC, or between the pre- and post-treatment Shannon indices for each sample. This suggests that treatment did not affect the diversity of species present, and the fact that the most abundant species, *C. catinatum*, was

poorly controlled by drug treatment goes a long way to explaining this. It also suggests that species diversity was not correlated with overall parasite egg shedding either. These data followed the general downward trend in nemabiome diversity over time observed in other studies.

In the four species which showed a consistent response to BZ treatment, there was no correlation between the change in species proportion and the overall reduction in FEC for that animal. This suggests that the reduction in FEC following anthelmintic treatment is not equal over all strongyle species, but varies with each horse owing to the significant differences in their nemabiomes. Prior determination of the nemabiome could therefore inform the likelihood of BZ treatment success in advance of administration, and reserve its use for animals whose parasite burden is composed of mainly susceptible species. Further work using different anthelmintic classes has the potential to build a valuable knowledge base which would allow targeted treatment with the most appropriate anthelmintic, and help delay the development of anthelmintic resistance.

5 APPLICATION OF FECPAK^{G2} AND NEMABIOME SEQUENCING TO EXOTIC EQUIDS

5.1 INTRODUCTION

Conservation of exotic equids by captive breeding can be essential, such as in the case of the Przewalski's horse (*Equus przewalskii*) which became extinct in the wild in the 1960s and has survived only as a consequence of a captive breeding programme (Souris *et al.*, 2007). Research has identified that exotic equids such as *E. przewalskii* and zebra species harbour intestinal helminth parasites of similar species to domestic equines (Epe *et al.*, 2009). Therefore, regular monitoring, such as the use of FECs, is a useful tool for helminth control in exotic equid species when in a captive environment, and may be the only intervention required for helminth control (Epe e*t al.*, 2009).

Free ranging wild zebra have typically higher worm burdens than do managed animals and in the absence of anthelmintic treatment are still commonly infected with large strongyles, which are now rare in domestic horse populations (Wambwa *et al.*, 2004). Commonly observed in both ranched and free ranging zebra is the nematode *Crossocephalus viviparous* from the family *Atractidae* which does not appear to infect horses despite their sharing of the same habitat (Krecek *et al.*, 1995) and is often found in high infection levels (Wambwa *et al.*, 2004). The majority of cyathostomin species infect all equids. However, there are some species particular to one host such as *Cylicocyclus triramosus* which is only known to infect zebra (Kharchenko *et al.*, 1997). Investigation using scanning electron microscopy has revealed that the ascarid species infecting both wild and captive zebra is the same one that infects the domesticated horse, namely *P. equorum* (Ansel *et al.*, 1974). Thus, the monitoring and understanding of parasitic helminth infections is a crucial part of exotic equid species conservation.

Following the successful optimisation and validation of the FECPAK^{G2} for use in equine samples (Chapters 2 and 3) and the use of the MinION to sequence nemabiomes from DNA extracted from equine faecal samples (Chapter 4), these methods were trialled on faecal samples from Chapman's zebra. Therefore, the aim of this work was to investigate the applicability of the FECPAK^{G2} and Nanopore sequencing as a method of understanding and monitoring parasitic helminth infections in exotic equids.

5.2 CHAPTER AIMS

- Explore FECPAKG2 and MinION sequencing suitability for exotic equids
- Examine nemabiome of zebra and compare to the equine nemabiomes sequenced

5.3 MATERIALS AND METHODS

5.3.1 Sample Collection and FECs

During the spring of 2018, samples were collected from a small herd of Chapman's zebra, *Equus quagga chapmani*, residing at Folly Farm Zoo and Adventure Park in Pembrokeshire, Wales UK. Individual samples were collected from three zebra, and a pooled sample was taken from two zebra (mare and foal) who were housed together. The samples were processed using the standard FECPAK^{G2} slide resistance testing protocol, described previously for equids (Section 7.2 of the Appendix). Following treatment with a BZ anthelmintic (Panacur [Intervet UK Ltd]; Fenbendazole active ingredient) administered in the feed, further samples from two of the zebra were obtained on day 14 after treatment, and processed using the same

method. In addition, the pre-treatment samples were imaged using the FECPAK^{G2} Micro-I using the optimised and validated equid protocol.

5.3.2 DNA extraction

DNA was extracted from two of the individual zebra pre-treatment samples which had observable infections from the positive identification of eggs during FECs for both strongyles and *Parascaris equorum*. DNA was extracted using the protocol described previously in Section 4.3.5 "DNA Extraction: CTAB (Cetyl Trimethylammonium Bromide) method" optimised for equine samples.

5.3.3 PCR amplification of zebra nemabiome ITS-2sequences

Amplicons were produced using the same method as in Section 4.3.9 "PCR of DNA extracted using CTAB method". Forward and reverse primers were taken from Avramenko *et al.* (2015). For PCR amplification, 25 µl reactions were performed using MyFi 2× DNA polymerase (Bioline, UK) according to the manufacturer's instructions with 10 µM forward and reverse primers and approximately 20 ng of zebra faecal DNA. A negative control was prepared using 1 µl Nuclease-free water, and a positive control using 20 ng equine faecal DNA. PCR conditions were: initial denaturation at 95ºC for 1 minute, denaturation at 95ºC for 15 seconds, annealing at 55ºC for 15 seconds, extension at 72ºC for 10 seconds and final extension at 72ºC for 5 minutes, with 35 cycles performed. PCR was repeated where necessary to provide sufficient template DNA for barcoding.

5.3.4 Barcoding zebra faecal DNA samples

Having determined in Chapter 4 (Section 4.3.13) that the barcodes to identify the individual nemabiome samples during the MinION sequencing process were best

used as the second stage of a nested PCR, a template was produced from each zebra sample, labelling each sample of amplified nematode ITS-2 rDNA produced from the zebra faecal samples. Two of the samples were selected for this work, those from Kanzi and Ayah, as they showed the highest FECs from the individual zebra faecal samples. In order to produce a sufficient quantity of the barcoded amplicons for MinION sequencing, three PCR runs for each sample were performed, using the individual barcoded primers number 27 (Kanzi) and 28 (Ayah) (refer to section 7.5 of the Appendix for barcodes). PCR conditions were as stated before in Section 5.3.3 "PCR amplification of zebra nemabiome ITS-2sequences", with repeat PCR runs as required.

5.3.5 MinION nemabiome sequencing

The barcoded zebra nemabiome amplified DNA samples were sequenced using the MinION desktop sequencer simultaneously with the horse samples as described previously in Chapter 4 (Section 4.3.14 MinION sequencing). Sequence quality control and sequence identification was performed on the resulting data as described in Chapter 4 (Section 4.3.15 Data analysis).

5.4 RESULTS

Given the success of FECPAK^{G2} development and MinION nemabiome sequencing of equine faecal samples, this chapter aimed to determine if anthelmintic resistance in the parasite nemabiome in a small population of zebra was present and to what extent their resident nemabiome differed from those in the domestic horse. Thus, zebra samples were monitored for their response to BZ anthelmintic treatment whilst the faecal samples were retained for future DNA analysis.

5.4.1 Sample Collection and FECs

Three out of the four samples demonstrated evidence of infection with both strongyles and *P. equorum* species (Table 5-1) with the highest epg recorded for strongyles at 189 epg. The highest recorded epg for *P. equorum* was the sample of 'Ayah' at 170 epg. For the two post-treatment zebra samples that were available, treatment with Fenbendazole (Panacur) gave a 100% reduction in the faecal egg counts (Table 5-1).

Table 5-1 Faecal egg counts (epg) using the FECPAK^{G2} slide on zebra samples. Pre- and Post-treatment FECs were performed using a BZ based anthelmintic.

	Pre-treatment		**Post-treatment**	
Name	**Strongyle epg**	***P. equorum* epg**	**Strongyle epg**	***P. equorum* epg**
Kanzi	28	83	0	0
Ayah	14	179	0	0
Nutmeg	18	0	-	-
Penny and Dayo	189	5	-	-

The pre-treatment samples were also imaged using the FECPAK^{G2} Micro-I which successfully visualised both strongyle and *P. equorum* eggs (Table 5-2).

Table 5-2 Mean of faecal egg counts (epg) using the FECPAK^{G2} slide (n=5) and FECPAK^{G2} cassette (n=2) on zebra faecal samples

	G2 slide		G2 cassette	
Name	**Strongyle epg**	***P. equorum* epg**	**Strongyle epg**	***P. equorum* epg**
Kanzi	28	83	0	0
Ayah	14	179	0	52
Nutmeg	18	0	13	0
Penny and Dayo	189	5	169	13

5.4.2 DNA extraction

DNA extracted from zebra faecal samples was imaged using gel electrophoresis on a 1% w/v Agarose gel run at 100 V to visualise the DNA. In total, 2 µl of zebra faecal DNA was loaded into each lane along with 0.5 µl 5 × loading dye (Bioline.com) (Figure 5-1).

It can be observed from Figure 5-1 that both DNA and RNA were present in the zebra faecal samples. Quantification of the DNA extracted from the zebra faecal samples using a NanoDrop spectrophotometer yielded nucleotide concentrations of 1164.9 ng/µl for Kanzi and 1471.9 ng/µl for Ayah. When quantified using a Qubit 2.0 Fluorometer, DNA concentrations of 200 ng/µl for Kanzi and 298 ng/µl for Ayah were obtained.

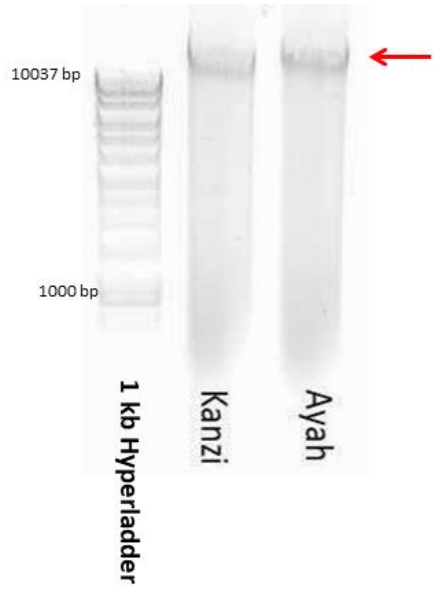

Figure 5-1 Zebra faecal DNA extraction using CTAB method analysed on a 1% agarose gel. The red arrow highlights high molecular weight DNA extracted, with the area beneath showing that RNA is also present.

5.4.3 PCR amplification of zebra nemabiome ITS-2 sequences

Nematode ITS-2 amplicons were produced from the DNA that had been extracted from two of the zebra pre-treatment faecal samples using the same method as in Chapter 4 (Section 4.3.9 PCR of DNA extracted using CTAB method). The PCR products were imaged using gel electrophoresis on a 1% w/v Agarose gel run at 100 V (Figure 5-2). A product of around 400 bp was produced from the DNA from the zebra faecal samples similar to that observed in the domestic equine samples, demonstrated by the positive control in Figure 5-2 which is an equine faecal DNA sample. In addition, a slightly smaller product was produced in the 'Kanzi' zebra sample, as had been observed in earlier work on equine faecal DNA samples. However, there were also several weaker products of smaller sizes produced from both zebra faecal DNA samples that had not been observed in the equine samples.

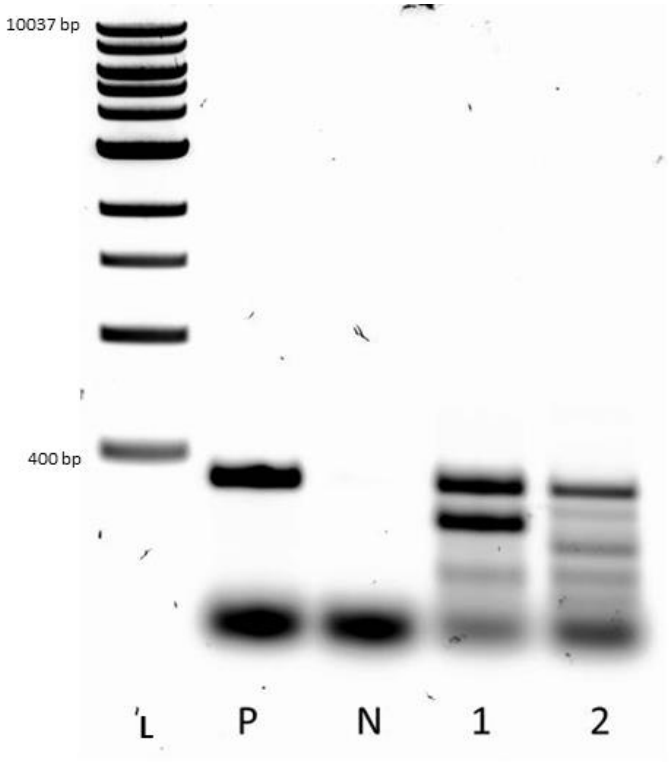

Figure 5-2 ITS-2 Amplicons from Zebra faecal DNA samples produced with MiFi 2× analysed on a 1% agarose gel. Lanes: L: 1kb Hyperladder, P: positive control (equine), N: negative control, 1: Kanzi, 2: Ayah)

5.4.4 Barcoding zebra faecal DNA samples

To produce the first nest of PCR prior to barcode labelling, the two zebra samples were amplified using the final PCR protocol (section 7.4 in the Appendix), and the products run on a 1% w/v Agarose gel. Initially, it did not appear that this process had been successful (Figure 5-3a). However, when the contrast was adjusted, faint bands could be observed Figure 5-3b).

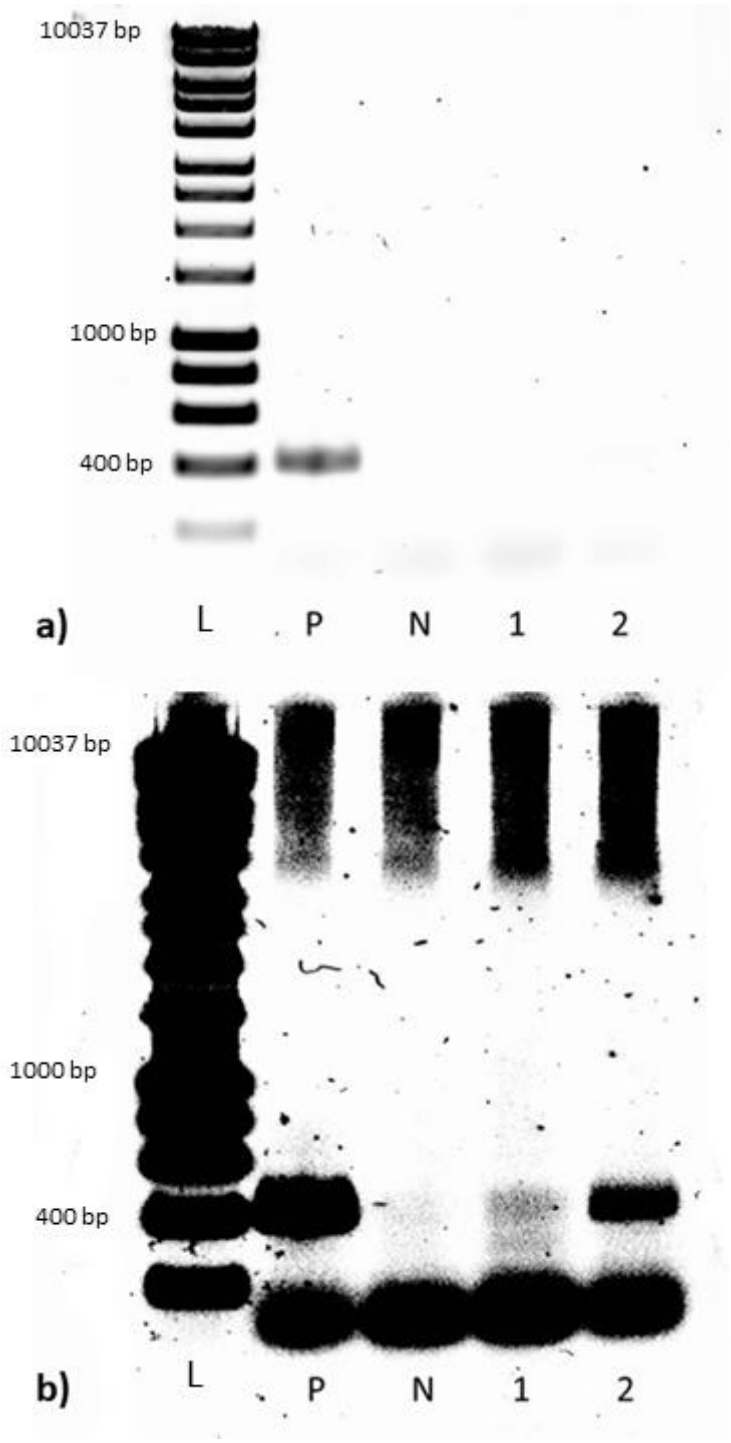

Figure 5-3 18S Amplicons from Zebra faecal DNA samples produced with MyFi 2× Mix analysed on a 1% agarose gel. Lanes: L: 1kb Hyperladder, P: positive control (equine), N: negative control, 1: Kanzi, 2: Ayah) a) with original contrast settings, b) with contrast adjusted to visualise products.

Despite low levels of PCR products, barcodes were added in a second nest of PCR as with the equine samples. Repeat PCR was performed, however products were again very weak (Data not shown).

5.4.5 MinION nemabiome sequencing

It was decided to take the zebra samples forward for nemabiome sequencing despite the low levels of PCR products generated, and the samples were sequenced using the MinION. The DNA sequences produced from the zebra faecal samples were subjected to the same DNA sequence quality control and identification procedures as used for the horse samples in Chapter 4 (Section 4.3.15 Data analysis). Following data analysis, the DNA sequences in the zebra samples representing the nemabiome could be grouped into five principal species, which coincided with the five most numerous species observed in the horse samples, namely *C. catinatum, C. pateratum, C. nassatus, C. goldi and C. longibursatus* (Figure 5-4). Interestingly, no DNA sequences were identified for *P. equorum* in common with the one horse sample that had shown a positive *P. equorum* egg count. Both zebra nemabiomes were similar to each other, with the exception of the proportions of *C. goldi* and *C. longibursatus,* which differed between the two samples.

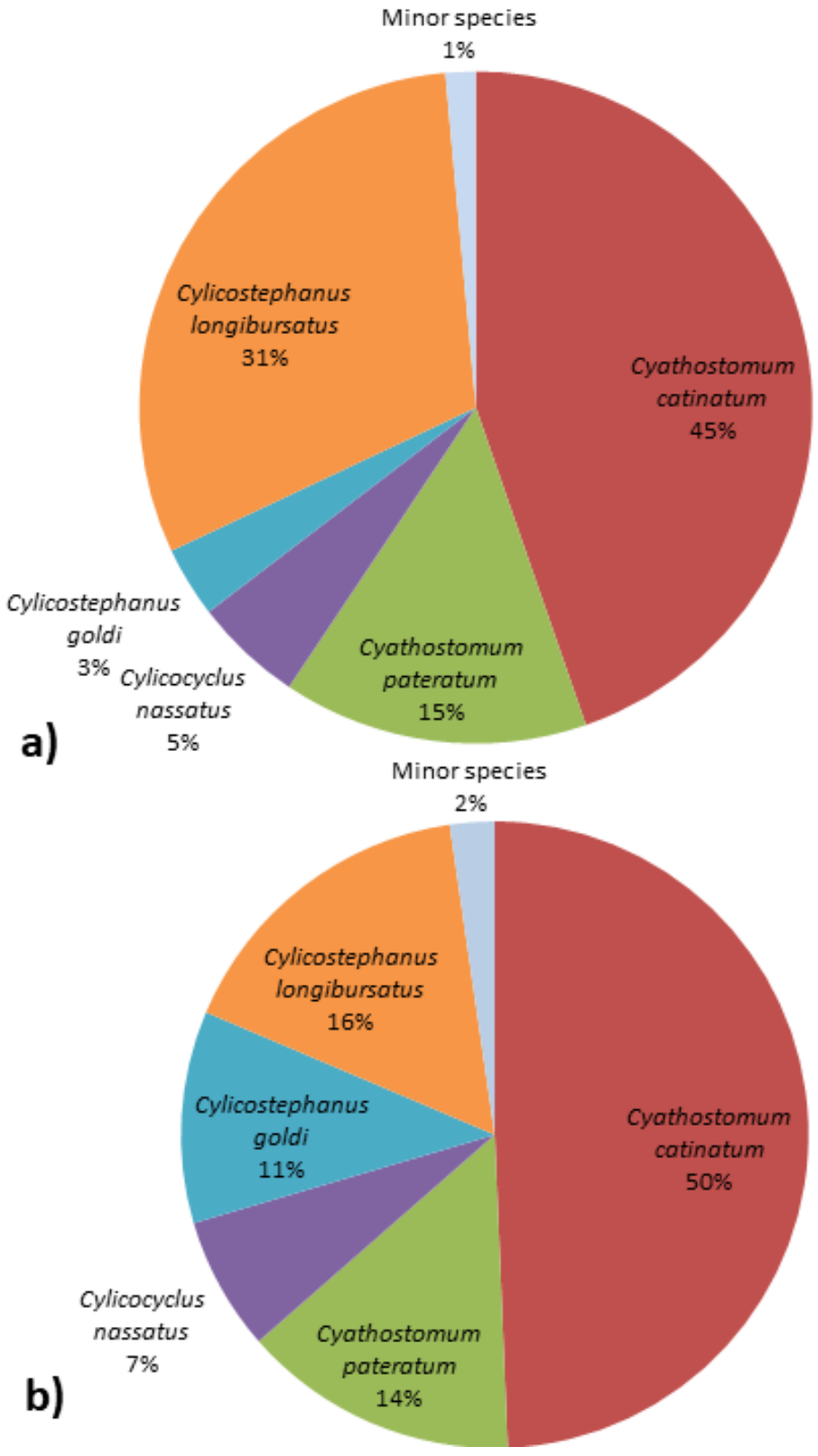

Figure 5-4 Proportions of DNA sequences identified by MinION sequencing in zebra pre-treatment faecal DNA samples a) Kanzi, b) Ayah. Five major cyathostomin sequences were identified, 'Minor species' represent species with less than 5% of DNA sequences in both nemabiomes.

5.5 DISCUSSION

Understanding and monitoring parasite infections in captive zebra is an important part of their husbandry, as management factors such as crowding, transfer of animals and stress due to factors such as weaning can precipitate temporary severe helminth infection (Epe *et al*., 2009). As many exotic equid species are endangered, it is especially important to avoid the development of anthelmintic resistance in order to safeguard captive populations. In exotic equids, non-invasive methods of monitoring levels of helminth infection and anthelmintic resistance are preferable; therefore the optimised FECPAK^{G2} and nemabiome sequencing techniques were expanded from domestic equines to alternative equid species.

When utilising FECs, three out of the four zebra faecal samples demonstrated evidence of *P. equorum* infection despite these samples being collected from adult animals. In domestic horses, immunity to *P. equorum* usually develops during the first eighteen months of life (Laugier *et al.*, 2012). However, a Kenyan study observed that 30% of adult Burchell's zebra, *Equus burchelli antiquorum* examined, both ranched and free ranging, harboured *P. equorum* infections (Wambwa *et al.*, 2004) suggesting that zebra do not develop immunity to *P. equorum* in the same manner as horses. This theory has also been suggested in relation to Grant's zebra, *Equus quagga boehmi*, with the author postulating that this might be a result of the zebra having not evolved alongside *P. equorum*, or that they are less resistant to it than are domestic equids (Scullion, 1982). A similar incidence of *P. equorum* infection (35%) to the Kenyan study was observed in a study of wild Grevy's zebra, *Equus grevyi* (Muoria *et al.*, 2005). In addition to *P. equorum*, the Kenyan study also identified *Strongylus* infections in 90% of the subjects, but cyathostomins in only 5%

(Wambwa *et al.*, 2004). A study of Chapman's zebra found a similar prevalence of *P. equorum* infection of 39%, and strongyle infection of 89% in German captive animals (Epe *et al.*, 2009). The zebra in the current study showed a higher prevalence of *P. equorum* infection than found in the literature in either captive or wild animals, although the number of animals monitored was small.

The post-treatment FECs on zebra samples demonstrated excellent success with a BZ based anthelmintic with a 100% reduction in both strongyle and *P. equorum* egg counts. The *P. equorum* result confirmed the findings from the horse samples tested previously (Chapter 4). In the current work, horse samples demonstrated widespread resistance to Fenbendazole in the population of strongyles yet this was not demonstrated in the zebra FECRTs. However, the work is not directly comparable as in the horse study, animals were only included in the equine study if the level of strongyle egg shedding before BZ treatment was at least 200 epg, and pre-treatment FECs in the zebra samples were much lower (28 epg and 14 epg in the two cases where paired pre- and post-treatment samples were available).

Performance of the FECPAK^{G2} Micro-I system was similar with zebra samples as with horse samples. The samples were imaged using the cassette only twice, compared to five times when using the FECPAK^{G2} slide, which gave a higher "lowest detection limit" when using the FECPAK^{G2} cassette and meant that the FECs of two of the samples fell below this limit and strongyle infections were thus not detected in those samples when using the cassette. *P. equorum* counts were highly variable between replicates of each sample when counting using the slide (e.g. FECs ranged from 23 epg to 161 epg in one sample, and 92 epg to 299 epg in another), meaning that the one time a *P. equorum* infection went unidentified when

using the FECPAK^{G2} cassette was likely due to a similar variability. It has been demonstrated that there is no linear relationship between FEC and worm burden for *P. equorum* in horses (Nielsen *et al.*, 2010), and thus strategic treatment against these parasites in horses is recommended (Rendle *et al*., 2019) with a positive FEC used as evidence of infection status rather than as a quantification of infection level. For zebra, regular FECs would therefore monitor the *P. equorum* infection status, and with replicate cassettes taking only seconds to prepare, greater accuracy at detecting helminth infections in zoo situations could easily be achieved. The FECPAK^{G2} system therefore represents a potentially useful tool for use with exotic equids.

Following FECRT analysis, DNA extractions were performed for application to nemabiome sequencing as demonstrated for the domestic equid. Initial success was demonstrated through PCR amplification of helminth DNA from the zebra faecal samples. Both of the faecal samples tested had low levels of strongyle infection (28 epg and 14 epg). However, both zebra had a reasonable level of *P. equorum* egg shedding at 83 epg and 179 epg respectively. Thus, it was hoped that subsequent sequencing might produce notable differences in nemabiome populations including the strongyle species present compared to the horse samples. However, with the increased presence of *P. equorum* infections in adult animals, unusual given domestic horses likely develop immunity by the age of twelve to eighteen months (Reinemeyer, 2009), it was hoped that sequencing data may provide evidence of *P. equorum* infection.

The strongyle species observed in the zebra nemabiome were, in fact, comprised of the same principal species as the domestic horse samples sequenced previously, albeit 5 principal species instead of 6. The missing principal species from the zebra

samples was that of *Coronocyclus coronatus* which was almost absent in the zebra faecal samples and thus was no longer classified as a principal species. However, this was the least numerous of the principal species in the equine samples at 2% of the total sequences, making its lower occurrence in the zebra samples likely due to the low overall strongyle FEC levels. Comparing the results of the zebra nemabiome DNA sequencing with the pre-treatment horse samples, the proportion of *C. catinatum* was approximately equal in proportions at around 50% along with the combined proportions of the two *Cylicostephanus* species at approximately a third of all sequences. However, in the zebra nemabiome there was a higher proportion of *C. pateratum* (15% as opposed to 4 – 10% in the three horse yards) and a much lower proportion of *C. nassatus* (5 – 7% as opposed to 13, 22 and 32% in the horse yards). Given that *C. pateratum* showed high levels of BZ resistance in the equine study, and *C. nassatus* was much better controlled, these differences could well be explained by Folly Farm's anthelmintic programme. The zebra in this study were regularly treated with BZ anthelmintics due to their method of administration being via feeding, an important advantage over syringe administered anthelmintics when dealing with essentially wild equid species (Lia *et al.*, 2010).

Compared to each other, the two zebra nemabiomes were very similar apart from the proportions of *C. goldi* and *C. longibursatus* identified. From the work on equine samples in Chapter 4 it was discovered that these two cyathostomin species exhibited an inconsistent response to BZ anthelmintic treatment, so repeated BZ use on the host zebra may have influenced this nemabiome difference. Access to faecal DNA samples from a greater number of zebra would be necessary for further exploration of the similarities between nemabiomes in this host species.

In common with the only identification of *P. equorum* within the horse samples was the absence of *P. equorum* DNA sequences within the nemabiome. One likely reason for this absence is that *P. equorum* eggs are protected by a tough shell unlike strongyle species (Kazacos and Turek, 1983), which may not have been successfully disrupted during the DNA extraction process. As yet, no research has been documented attempting to extract DNA from *P. equorum* eggs. Alternative research has looked at DNA extracted from *P. equorum* adults themselves (Janssen *et al.*, 2013; Tydén *et al.*, 2013) or, where eggs were used, by performing a larval culture and then extracting the DNA from the larvae produced (Tydén *et al.*, 2014). The inability of *P. equorum* DNA to be extracted during the current work, to incorporate *P. equorum* into the nemabiome analysis, was disappointing from a view point of completeness. However, as the eggs of *P. equorum* are readily identified visually during the FEC process, incorporation into DNA sequencing analysis for potential diagnostics is of limited interest. Although a larval hatch assay would allow more complete sequencing of the nemabiome, the process is far more time consuming and labour intensive than faecal DNA extraction, therefore a combination FEC and faecal DNA sequencing approach is potentially far more scalable for routine use.

5.6 CONCLUSION

The work performed for this chapter demonstrates that both the FECPAK^{G2} and MinION nemabiome sequencing were effective for zebra. It also illustrated that the cyathostomin nemabiome sequencing method was effective even at low FEC levels. These methods therefore represent useful tools to improve the health of captive zebra, and could also be used to monitor that of zebra in the wild.

The zebra in this study harboured the same species of cyathostomin as had been found in the equine samples. However, there was a greater proportion in the zebra samples of those cyathostomin species that had shown the greatest resistance to BZ treatment in the equine study. This suggests that the history of repeated BZ administration in the zebra used in the study may have influenced the host nemabiomes towards those species least responsive to BZ treatment. Therefore, monitoring of the change in zebra nemabiomes over time, together with FEC monitoring, would give an early warning if treatment with an alternative anthelmintic would be advisable to prevent problematic levels of helminth infections occurring.

6 FINAL DISCUSSION

6.1 DEVELOPMENT AND VALIDATION OF THE FECPAK^{G2} FOR EQUIDS, AND MONITORING OF ANTHELMINTIC RESISTANCE

The work in Chapters 2, 3 and 5 demonstrated that the FECPAK^{G2} could be applied to the equid sector, both domestic equines and exotic species, with some modification of the initial preparation of the faecal samples compared to ruminant samples. The FECPAK^{G2} therefore offers a user-friendly method of monitoring helminth infections in these host species. Sustainable control of equine helminths requires monitoring of egg shedding via FECs (Matthews, 2014) yet studies demonstrate that the majority of horse owners do not utilise FECs at all, or do not perform them with the recommended frequency (Slater, 2017). Furthermore, the potential for a more sensitive test with a lower detection limit exists given the challenges discovered during this work associated with the optics of the FECPAK^{G2} Micro-I. Therefore, planned improvements to the imaging system will no doubt lead to a user friendly and sensitive diagnostic. Availability of such diagnostics may improve the current situation, wherein even Veterinary practices often recommend anthelmintic treatment without prior diagnosis of infection (Sallé and Cabaret, 2015), and would be of great benefit if the Danish system, whereby equine anthelmintics are obtainable only in cases where clinical diagnosis of infection is present (Nielsen *et al.*, 2014b), was emulated in other countries.

Additionally, the opportunity exists for further development of the FECPAK^{G2} for additional uses. Further optimisation work similar to that undertaken in Chapter 2 offers the potential for expansion into other species where GI parasites are also problematic, both domestic and exotic. In the absence of FEC testing,

recommendation is for anthelmintic treatment to be administered monthly to dogs under some management regimes (ESCCAP, 2017), offering the potential for substantial reductions in anthelmintics if FECs were routinely used.

Development of the FECPAK^{G2} for other exotic species offers the potential for monitoring and control of helminth parasites in zoos and wildlife parks, where widespread infection of GI helminths is often detected (Goossens *et al.*, 2005; Lim *et al*., 2008). Further work could thus support the conservation of many endangered species. Furthermore, potential exists for the development of the FECPAK^{G2} for the detection of additional parasite species in addition to nematodes. Ongoing work to adapt the cassette system to sediment eggs rather than float them offers the potential for diagnosis of fluke infections (Reigate, unpublished data), although there remain some parasite species for which FEC is not a suitable diagnosis, such as *Anoplocephala perfoliata* (Meana *et al*., 1998).

Significant anthelmintic resistance was detected against BZs in the equine cyathostomin populations monitored, although BZs still appeared to be effective against *P. equorum* in both the domestic equines and zebra tested. Strongyle FECs in the zebra were much lower than in the equine samples, and were effectively controlled by BZ in the host zebra monitored. A larger population of zebra to study would inform if the apparent efficacy of BZ was due to the low FEC levels, or vice versa. Continued and expanded monitoring could inform the treatment decision support offered by Techion Ltd. when the FEC results are returned. The availability of large amounts of equine FEC data, which will be collected if the FECPAK^{G2} system becomes widely utilised, offers the potential for early warning if a particular anthelmintic compound starts showing reduced efficacy. Feeding this information

back into future research will likely improve the quality of the decision support, and may offer some scope to investigate the efficacy of different treatment thresholds, as the current recommendation of treatment at 200 epg (Coles, 2009) is still only arbitrary. Of course, research into decision support would be needed for additional host species, especially exotics where little research currently exists.

6.2 OPPORTUNITIES WITH NEMABIOME SEQUENCING

Gastro-intestinal helminths live in complex communities within the host (Lello *et al.*, 2004) and little research has been done studying these communities within equine hosts (Mitchell *et al.*, 2019). The work performed in Chapter 4 optimised a DNA extraction and PCR protocol which allowed strongyle DNA to be extracted from eggs within equine faecal samples, which could then be used for amplification of the ITS-2 region followed by sequencing using a MinION benchtop sequencing device in order to determine the equine 'nemabiome' pre and post anthelmintic exposure. When performed on the zebra faecal samples in Chapter 5 it demonstrated that this approach remained successful even at low egg shedding levels, with their consequent low levels of DNA. This finding offers promise that DNA extracted directly from the FECPAK^{G2} cassette preparations could be successfully sequenced, in order that nematode eggs could be both visualised and identified to species level. Nanopore sequencing of microbial populations has been carried out entirely off-grid and in remote locations (Edwards *et al.*, 2018; Gowers *et al.*, 2019) suggesting that nemabiome sequencing could also likely be used in remote areas to support wild or semi-managed populations of exotic equids and potentially other species.

Importantly, differential diagnosis of large or small strongyle infections is possible using faecal DNA extraction and nemabiome sequencing. There is some evidence that *S. vulgaris* is re-emerging due to TST essential to reduce anthelmintic resistance in cyathostomins (Nielsen *et al.*, 2012). As *S. vulgaris* is more pathogenic than small strongyle species (Pihl *et al*., 2017) a simple, non-invasive test to monitor for the presence of *S. vulgaris* is an important weapon in the fight against equid GI nematodes.

The nemabiome investigation in Chapter 4 demonstrated that BZ resistance is not uniform across cyathostomin species, but likely varies depending on their preferred site in the GI tract. Those species which reside most caudally in the GI tract were least well controlled by the administration of BZ anthelmintics, whereas those species with a predilection for more cranial areas of the GI tract did not exhibit consistent resistance to treatment. Species diversity was not affected by a single BZ treatment, but the species diversity of nemabiomes in the current study followed a general trend towards decreasing diversity over time. This reduction was further confirmed investigating zebra nemabiomes (Chapter 5) which exhibited the same principal species as those found in the equine study, but with the proportions skewed towards those more resistant to BZ treatment, possibly as a result of their history of repeated BZ exposure and limited rotational grazing opportunities.

The variable overall FEC reduction following BZ treatment observed in the current equine study appeared to be caused by the differing host nemabiomes, and consequently nemabiome sequencing prior to treatment offers crucial information as to whether BZ treatment is likely to be successful, in advance of its administration. Therefore, continuation of the current nemabiome methodology could support and

potentially determine the susceptibility of cyathostomin species to alternative classes of anthelmintic. Given the level of non-BZ anthelmintic resistance (Peregrine *et al.*, 2014; Tzelos *et al.*, 2017) such an approach could lead to a tailored treatment approach in a manner akin to that currently employed with targeted antibiotic treatment (Viceconte *et al.*, 2017). Any information acquired prior to anthelmintic usage to select an appropriate anthelmintic would potentially further delay the development of anthelmintic resistance by avoiding exposing helminth species to anthelmintic compounds that do not adequately control them.

Expanding the current equine nemabiome monitoring process across the UK would potentially inform whether resistant cyathostomin species are evenly distributed across the country or, more likely, if there are clusters in different locations representing differing BZ resistance phenotypes (Hodgkinson *et al.*, 2008; Ishii *et al.*, 2017). In doing so, nemabiome monitoring could offer the potential of preserving populations of equines where BZ anthelmintics, and potentially also other anthelmintic classes, are still effective by avoiding introducing those cyathostomin species least responsive to treatment (Leathwick *et al.*, 2019). In addition, studying populations of alternative equid host species such as donkeys and mules (and a greater number of zebra) would also be interesting to determine if the nemabiomes differ with different host species, or if the changes are more highly correlated with management practices and their geographical location. Moreover, the addition of nemabiome data to existing modelling techniques such as those used by Sauermann *et al*. (2019) would enhance our understanding of resistant cyathostomin epidemiology.

Further adaptation of molecular techniques and Nanopore sequencing offers the potential to detect mutations in SNPs associated with BZ resistance in cyathostomins (Hodgkinson *et al*., 2008), and monitoring populations of parasites in this way would track the spread of BZ resistant alleles. This need not be limited to equine parasites, and could be expanded to other anthelmintic classes and other host species where anthelmintic resistance is documented (James *et al.*, 2009) especially those where more complete helminth genomes are available for reference.

6.3 POTENTIAL OF NEXT GENERATION SEQUENCING AND METAGENOMICS

The potential exists to monitor the nemabiomes of numerous host animals, but the power of this approach is not limited to merely monitoring helminth populations. A combination of the two techniques discussed above offers the potential to identify SNPs associated with anthelmintic resistance and combine this information with the change in nemabiome following anthelmintic treatment. More complete and improved quality genomes exist for nematode parasites of sheep and cattle (https://parasite.wormbase.org/species.html), which would likely make this approach more immediately successful in host species other than equines.

Metagenomic analysis, which can assemble genomes from mixed samples of DNA using sophisticated bioinformatics tools, offers the future possibility of discovering more about the structure and function of genes in mixed populations of helminths, in addition to exploring the nemabiomes themselves (Roumpeka *et al.*, 2017). This technique has already been used on DNA from faecal samples, albeit hitherto only for microbial populations (Alneberg *et al.*, 2014). As metagenomics techniques are

further developed for eukaryotes, the potential to analyse cyathostomin DNA using the whole-genome-shotgun techniques could be applied to pre- and post-anthelmintic treatment faecal DNA samples, and therefore offer an insight into the mode of IVM resistance which is currently poorly understood (Laing *et al*., 2017). This approach could additionally add to our library of cyathostomin genomes, which are woefully under-represented in current databases.

6.4 LIMITATIONS OF THE WORK CONDUCTED

Validation of the more sensitive FECPAK^{G2} protocol was hampered by the problems with the image stacking software, however once this issue is rectified it should be possible to repeat the validation work for the 25 ml slurry protocol. In addition, other egg recovery methods could be investigated, potentially validating the system for use with a much lower multiplication factor and hence lower limit of detection.

An omission when monitoring the nemabiome changes was the monitoring the nemabiome of a horse or horses as a negative control, determining the extent of change in the nemabiome over a two week period without an anthelmintic being administered. This would have been an interesting comparison to the changes seen in the nemabiomes after BZ administration.

6.5 FURTHER DEVELOPMENT OF THE WORK

As mentioned in section 6.4, monitoring the changes in nemabiomes over time of horses without anthelmintic treatment would be interesting. A longitudinal study, sampling and sequencing every two weeks could track potential changes in the species present. In addition, the study could be repeated to monitor the nemabiomes of horses treated with other anthelmintics, although the samples would need to be

taken at a greater interval than two weeks post treatment, to allow re-infection to occur. This work would inform if certain species are showing a reduced egg reappearance period (ERP) which is a precursor to resistance developing (Shea Porr *et al.*, 2017).

A larger study of nemabiome monitoring could detect whether there are geographical differences in the helminth species present, as the current study had too few samples from each area studied. This would not necessarily need to be horses with a FEC of over 200 epg as treatment would not be needed for pure nemabiome monitoring, which would ameliorate the lack of samples due to low FECs that hampered this study.

6.6 RECOMMENDATIONS FOR COMMERCIAL USE

An immediate commercial application for the work in this study is the use of the now validated FECPAK^{G2} system as a FEC monitoring system for equids. In addition, the optimisation and validation work could be repeated for other host species, both domestic and exotic, to expand the range of usefulness of the system.

In premises where cyathostomin infection is adequately controlled with a system of FEC testing and pasture management, there is the potential for large strongyles such as *S. vulgaris* to become problematic once again (Nielsen *et al.*, 2012). DNA extraction and sequencing from faecal samples could offer a differential diagnosis to monitor whether these parasites are present, which would offer peace of mind that a zero anthelmintic regime is not storing up potential problems for the future.

6.7 CONCLUSION

In conclusion, the work performed hitherto has developed the FECPAK^{G2} for use in equids, which will support TST in the future for helminth control. A pipeline has been established for monitoring both equid FECs and changes in their nemabiomes, with both techniques highly portable and therefore usable in the field for both domestic and exotic equids. Given these developments, despite the absence of additional anthelmintic compounds, the future of cyathostomin control looks more promising.

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7 APPENDIX

7.1 STANDARD FECPAK G1 PROTOCOL

Throughout the optimisation and validation work, control values were obtained by performing a number of FECPAK^{G1} (G1) counts on the slurry and taking the mean value. The protocol is described below:

1. Dilute sample in a 1:4 ratio with water in a Ziploc bag and mix until a uniform slurry is produced.
2. Pour the slurry into the G1 measuring cylinder up to the 45 ml mark.
3. Add saturated NaCl solution was added up to the 230 ml mark, and stopper the cylinder.
4. Invert three times to mix, and then pour approximately half of the resulting liquid through a dampened 670 µm sieve into a jug.
5. Fill a FECPAK slide, using a Pasteur pipette, with an aliquot of this liquid.
6. Count using a microscope at 40x magnification, each egg represents 25 epg.

7.2 STANDARD RESISTANCE TESTING PROTOCOL

During the optimisation phase, a protocol for resistance testing was developed, which gives a sensitivity of 4.6 epg. This protocol is described below:

1. Dilute sample in a 1:4 ratio with water in a Ziploc bag and mix until a uniform slurry is produced.
2. Scoop 20 ml slurry into a FECPAK sedimentor, top up with water.
3. Pre-filter this liquid by pouring into a FECPAK cylinder fitted with a 1,000 µm (white) filter, and invert three times to mix.
4. Pour the liquid back into the sedimentor through the hole in the top of the cylinder, and leave to sediment for 30 minutes.
5. Discard the supernatant and dilute the sediment with 80 ml of saturated NaCl solution.
6. Transfer into a FECPAK cylinder fitted with 425 µm and 250 µm (black) filters.
7. Fill 5 FECPAK slides from the centre hole in the cylinder using a Pasteur pipette, inverting to mix between sub-samples.
8. Count using a microscope at 100 × magnification. On each slide, one egg represents 23 epg. Final FEC is the average of the five slides (sensitivity 4.6 epg).

7.3 CTAB DNA EXTRACTION PROTOCOL

1. Place the sample in a 2 ml tube. To each tube, add one 1.5 – 2 mm glass bead, and 600 µl lysis buffer.
2. Bead-beat at 50 bps for 3 minutes, using the Tissue Lyser (QiAgen), and spin briefly to collect the material at the bottom of the tube.
3. Incubate at 95ºC for 10 minutes.
4. Centrifuge at 500 × g for 1 minute.
5. To each tube, add 60 µl 3M potassium acetate, and mix gently by inversion.
6. Incubate on ice for 5 minutes.
7. Centrifuge at 17,000 × g for 5 minutes.
8. Recover 400 µl liquid into new 2 ml tubes (or as much as possible if there isn't 400 µl)
9. To each tube, add 50 µl 5M NaCl and 50 µl CTAB/NaCl pre-heated to 65ºC by microwaving on full power for 30 seconds.
10. Vortex to mix.
11. Incubate at 60ºC for 5 minutes, vortex and incubate for a further 5 minutes.
12. To each tube, add 300 µl Chloroform:Isoamyl alcohol 24:1, and mix well to form an emulsion.
13. Centrifuge at 17,000 × g for 5 minutes.
14. Transfer 300 µl of the supernatant into a new 1.5 ml tube add 300 µl isopropranol, and mix by inversion.
15. Centrifuge at 17,000 × g for 5 minutes, and discard the supernatant.
16. Wash each pellet with 500 µl fresh 70% ethanol and centrifuge at 17,000 × g for 1 minute. Repeat.

17. Discard the ethanol and dry the pellets at room temperature in a fume hood for two to three hours.
18. To each tube, add 50 µl nuclease-free water and incubate on ice for 30 minutes.
19. Vortex to resuspend the DNA.
20. Measure DNA quantity and purity using a Nanodrop.

7.4 FINAL PCR PROTOCOL

Positive control:

1 - 4 µl Template (depending on what's being used)

1 µl Forward primer (Avramenko)

1 µl Reverse primer (Avramenko)

13 µl MyFi 2x mix (Bioline.com)

Nuclease-free water to 25 µl

Negative control:

1 µl Forward primer (Avramenko)

1 µl Reverse primer (Avramenko)

13 µl MyFi 2x mix (Bioline.com)

10 µl Nuclease-free water

Samples and zero FEC negative control:

1 µl Template

1 µl Forward primer (Avramenko)

1 µl Reverse primer (Avramenko)

13 µl MyFi 2x mix (Bioline.com)

9 µl Nuclease-free water

Appendix

PCR conditions:

Initial denaturation	95ºC	1 minute		
Denaturation	95ºC	15 seconds	}	
Annealing	55ºC	15 seconds	}	35 cycles
Extension	72ºC	10 seconds	}	
Final extension	72ºC	5 minutes		

7.5 BARCODE PRIMERS

Table 7-1 Barcodes allocated to each faecal DNA sample

DNA Sample	Barcode
Widget pre	1
Widget post	2
Klettur pre	3
Klettur post	4
Major pre	5
Major post	6
Gertie pre	7
Gertie post	8
Seren pre	9
Seren post	10
Jigsaw pre	11
Jigsaw post	12
Hope pre	13
Hope post	14
Billee pre	15
Billee post	16
Duke pre	17
Duke post	18
Sox pre	19
Sox post	20
Topaz pre	21
Topaz post	22
Mary pre	23
Mary post	24
Yoyo pre	25
Yoyo post	26
Kanzi	27
Ayah	28

BC	Leader	BC	Primer sequence		Full
BC01	ATCGCCTACCGTGAC	AAGAAAGTTGTCGGTGTCTTTGTG	ACGTCTGGTTCAGGGTTGTT		ATCGCCTACCGTGACAAGAAAGTTGTCGGTGTCTTTGTGACGTCTGGTTCAGGGTTGTT
BC02	ATCGCCTACCGTGAC	TCGATTCCGTTTGTAGTCGTCTGT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACTCGATTCCGTTTGTAGTCGTCTGTACGTCTGGTTCAGGGTTGTT
BC03	ATCGCCTACCGTGAC	GAGTCTTGTGTCCCAGTTACCAGG	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGAGTCTTGTGTCCCAGTTACCAGGACGTCTGGTTCAGGGTTGTT
BC04	ATCGCCTACCGTGAC	TTCGGATTCTATCGTGTTTCCCTA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACTTCGGATTCTATCGTGTTTCCCTAACGTCTGGTTCAGGGTTGTT
BC05	ATCGCCTACCGTGAC	CTTGTCCAGGGTTTGTGTAACCTT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCTTGTCCAGGGTTTGTGTAACCTTACGTCTGGTTCAGGGTTGTT
BC06	ATCGCCTACCGTGAC	TTCTCCGCAAAGGCAGAAAGTAGTC	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACTTCTCCGCAAAGGCAGAAAGTAGTCACGTCTGGTTCAGGGTTGTT
BC07	ATCGCCTACCGTGAC	GTGTTACCGTGGGAATGAATCCTT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGTGTTACCGTGGGAATGAATCCTTACGTCTGGTTCAGGGTTGTT
BC08	ATCGCCTACCGTGAC	TTCAGGGAACAAACCAAGTTACGT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACTTCAGGGAACAAACCAAGTTACGTACGTCTGGTTCAGGGTTGTT
BC09	ATCGCCTACCGTGAC	AACTAGGCACAGCGAGTCTTGGTT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACAACTAGGCACAGCGAGTCTTGGTTACGTCTGGTTCAGGGTTGTT
BC10	ATCGCCTACCGTGAC	AAGCGTTGAAACCTTTGTCCTCTC	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACAAGCGTTGAAACCTTTGTCCTCTCACGTCTGGTTCAGGGTTGTT
BC11	ATCGCCTACCGTGAC	GTTTCATCTATCGGAGGGAATGGA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGTTTCATCTATCGGAGGGAATGGAACGTCTGGTTCAGGGTTGTT
BC12	ATCGCCTACCGTGAC	CAGGTAGAAAGAAGCAGAATCGGA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCAGGTAGAAAGAAGCAGAATCGGAACGTCTGGTTCAGGGTTGTT
BC13	ATCGCCTACCGTGAC	AGAACGACTTCCATACTCGTGTGA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACAGAACGACTTCCATACTCGTGTGAACGTCTGGTTCAGGGTTGTT
BC14	ATCGCCTACCGTGAC	AACGAGTCTCTTGGGACCCATAGA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACAACGAGTCTCTTGGGACCCATAGAACGTCTGGTTCAGGGTTGTT
BC15	ATCGCCTACCGTGAC	AGGTCTACCTCGCTAACACCACTG	ACGTCTGGTTCAGGGTTGTT		ATCGCCTACCGTGACAGGTCTACCTCGCTAACACCACTGACGTCTGGTTCAGGGTTGTT
BC16	ATCGCCTACCGTGAC	CGTCAACTGACAGTGGTTCGTACT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCGTCAACTGACAGTGGTTCGTACTACGTCTGGTTCAGGGTTGTT
BC17	ATCGCCTACCGTGAC	ACCCTCCAGGAAAGTACCTCTGAT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACACCCTCCAGGAAAGTACCTCTGATACGTCTGGTTCAGGGTTGTT
BC18	ATCGCCTACCGTGAC	CCAAACCCAACAACCTAGATAGGC	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCCAAACCCAACAACCTAGATAGGCACGTCTGGTTCAGGGTTGTT
BC19	ATCGCCTACCGTGAC	GTTCCTCGTGCAGTGTCAAGAGAT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGTTCCTCGTGCAGTGTCAAGAGATACGTCTGGTTCAGGGTTGTT
BC20	ATCGCCTACCGTGAC	TTGCGTCCTGTTACGAGAACTCAT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACTTGCGTCCTGTTACGAGAACTCATACGTCTGGTTCAGGGTTGTT
BC21	ATCGCCTACCGTGAC	GAGCCTCTCATTGTCCGTTCTCTA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGAGCCTCTCATTGTCCGTTCTCTAACGTCTGGTTCAGGGTTGTT
BC22	ATCGCCTACCGTGAC	ACCACTGCCATGTATCAAAGTACG	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACACCACTGCCATGTATCAAAGTACGACGTCTGGTTCAGGGTTGTT
BC23	ATCGCCTACCGTGAC	CTTACTACCCAGTGAACCTCCTCG	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCTTACTACCCAGTGAACCTCCTCGACGTCTGGTTCAGGGTTGTT
BC24	ATCGCCTACCGTGAC	GCATAGTTCTGCATGATGGGTTAG	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGCATAGTTCTGCATGATGGGTTAGACGTCTGGTTCAGGGTTGTT
BC25	ATCGCCTACCGTGAC	GTAAGTTGGGTATGCAACGCAATG	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACGTAAGTTGGGTATGCAACGCAATGACGTCTGGTTCAGGGTTGTT
BC26	ATCGCCTACCGTGAC	CATACAGCGACTACGCATTCTCAT	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCATACAGCGACTACGCATTCTCATACGTCTGGTTCAGGGTTGTT
BC27	ATCGCCTACCGTGAC	CGACGGTTAGATTCACCTCTTACA	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACCGACGGTTAGATTCACCTCTTACAACGTCTGGTTCAGGGTTGTT
BC28	ATCGCCTACCGTGAC	TGAAACCTAAGAAGGCACCGTATC	ACGTCTGGTTCAGGGTTGTT	-	ATCGCCTACCGTGACTGAAACCTAAGAAGGCACCGTATCACGTCTGGTTCAGGGTTGTT

7.6 12 ML VALIDATION FIGURES

Table 7-2 Validation results, 12 ml protocol showing mean of G1 control counts (four per horse) and mean of G2 Micro-I counts (four per horse). Notes show which results were included in the mean percentage accuracies.

Horse	G1 epg	G2 epg	G2 % of G1	Notes
Billee	606	505	83	sedimentor
Wellie	212.5	193.5	91	sedimentor
Dafydd	237.5	193.5	81	sedimentor
Buddy	662.5	791	119	sedimentor
Holly	875	722	83	sedimentor
Meg	663	456	69	sedimentor
Jack	794	823	104	sedimentor
Lily	850	946	111	sedimentor
Phoebe	1769	2150	122	sedimentor
Bronwen	219	312	142	sedimentor
Gola	331	269	81	sedimentor
Fluga	244	161	66	sedimentor
Topaz	1713	1527	89	sedimentor
Yoyo	394	344	87	sedimentor
Linus	163	194	119	sedimentor
Diplomat	394	151	38	old
Buddy	244	97	40	old
Sven	269	108	40	old
Imogen	113	11	10	old
Onyx	588	462	79	old
Thomas	138	97	70	old
Rose	600	344	57	old
Seren	213	129	61	old
Polly	700	301	43	old
Sam	738	602	82	sedimentor
Flyer	188	194	103	sedimentor
Monarch	413	215	52	scoop
Brooklyn	456	409	90	scoop
Ted	406	215	53	scoop
Mundana	300	108	36	scoop
Dusty	288	204	71	scoop
Sioned	613	516	84	scoop
Blackberry	131	118	90	scoop
Don	1413	1011	72	scoop
Fudge	625	58	58	scoop
Cadno	300	129	43	scoop
Harry	1125	570	51	scoop
Petra	788	796	101	scoop

Napoleon	356	301	85	scoop	
Ellie	675	430	64	scoop	
Mean percentage accuracy measured with sedimentor					96
Mean percentage accuracy measured with scoop					68

7.7 REPEAT IMAGING OF CASSETTES

Table 7-3 Repeat images from cassettes obtained using timed capture software. Eight repeat images were produced for each cassette, the numbers represent the eggs seen in each of the two wells for each consecutive image produced.

Sample 1 20/6/17		Sample 2 20/6/17		Sample 1 7/9/17		Sample 2 7/9/17		Sample 3 7/9/17	
Well 1	**Well 2**	**Well 1**	**Well 2**	**Well 1**	**Well 2**	**Well 1**	**Well 2**	**Well 1**	**Well 2**
0	6	0	0	5	2	2	7	4	4
0	5	0	1	5	2	2	7	4	4
1	3	0	3	5	3	2	7	4	4
1	5	0	4	5	3	2	7	4	4
1	5	0	1	5	3	2	6	4	4
0	5	0	4	5	3	2	7	4	4
0	5	0	4	5	3	2	7	4	4
0	5	0	4	5	2	2	7	4	4

Sample 4 7/9/17		Sample 5 7/9/17		Sample 6 7/9/17		Sample 1 13/9/17		Sample 1 14/9/17	
Well 1	**Well 2**	**Well 1**	**Well 2**	**Well 1**	**Well 2**	**Well 1**	**Well 2**	**Well 1**	**Well 2**
1	2	1	1	1	0	4	2	4	3
0	2	1	1	1	0	4	2	6	3
0	2	1	1	1	0	4	2	6	3
0	2	1	1	1	0	4	2	7	3
0	2	1	1	1	0	4	2	7	3
0	2	2	1	1	0	4	2	7	3
0	2	2	1	1	0	4	2	7	3
0	2	2	1	1	0	4	2	8	3